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Angiopoietin-2 and VEGF in non-small cell lung cancer prognosis: can vessel co-option have a role here?

Ana Luísa Pequeno Coelho

Dissertação de candidatura ao grau de Doutor em Biomedicina

submetida à Faculdade de Medicina da Universidade do Porto

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LIST OF PUBLICATIONS

According to the line a) of the 31st article of the Decree-Law nº 230/2009, the present Thesis has already produced the following publications in scientific peer-reviewed journals:

Coelho AL, Gomes MP, Catarino RJ, Rolfo C, Lopes AM, Medeiros RM, Araújo AM. "Angiogenesis in NSCLC: is vessel co-option the trunk that sustains the branches?"Oncotarget. 2016 Feb 29. [Epub ahead of print]

Coelho AL, Araújo AM, Gomes MP, Catarino RJ, Andrade EB, Lopes AM, Medeiros RM. "Combined Ang-2 and VEGF serum levels: holding hands as a new integral biomarker in non-small-cell lung cancers." Future Oncol.2015;11(24):3233-42.

Coelho AL, Araújo A, Gomes M, Catarino R, Marques A, Medeiros R. "Circulating Ang-2 mRNA expression levels: looking ahead to a new prognostic factor for NSCLC." PLoS One. 2014 Feb 28;9(2).

I, hereby declare that I actively participated in the gathering and study of the material included in each of the publications presented and wrote the manuscripts in collaboration with the other authors.

ABSTRACT

Introduction: Lung cancer remains the most common incident form of cancer globally, with deaths exceeding those from any other type of malignancy, accounting for nearly one in five deaths. Approximately 85% of those cases are non-small-cell lung cancers (NSCLCs) and the vast majority of patients presents at advanced stages of disease, resulting in an overall five-year survival around 15.9%. Lung cancer is the result of a multistep process, in which angiogenesis assumes a major role, allowing tumor access to oxygen and nutrients, growth factors and hormones. Antiangiogenic strategies are becoming widely used in NSCLC treatment and the pathway involving Vascular Endothelial Growth Factor (VEGF) and its receptors (VEGFRs) is the main target of the approved antiangiogenic molecules (bevacizumab and ramucirumab). Nevertheless, the overall survival (OS) benefits from these therapies are modest, because a high fraction of tumors is intrinsically refractory to the therapy and a substantial proportion of the remaining acquire resistance during treatment. Intensive research in this field unveiled some of the mechanisms underneath these disappointing results. These include: 1) upregulation of alternative pro-angiogenic signalling circuits to overcome VEGF(R) inhibition; 2) recruitment of vascular progenitor cells and pro-angiogenic monocytes from the bone marrow and 3) alternative mechanism to angiogenesis during tumor development, such as vessel co-option. Another obstacle in the quest for successful antiangiogenic strategies is the lack of reliable predictive biomarkers that may help to tailor patients for whom these therapies will be more suited. Angiopoietin-2 (Ang-2), a vessel destabilizing cytokine expressed by the endothelial cells, that belongs to the angiopoietin/Tie (type I transmembrane tyrosine kinase receptors) axis, works in concert with VEGF in tumor angiogenesis, and is now one of the most promising therapeutic targets in antiangiogenic strategies. It seems to be largely involved in the proposed escape mechanisms to current antiangiogenics and has a major advantage over VEGF, since its expression is seldom detected in healthy vasculature and highly regulated at mRNA level. These particular characteristics also turn it into an interesting candidate as a predictive and prognostic biomarker of outcome in antiangiogenic directed therapies.

Aims: Evaluation of the correlation between circulating Ang-2 mRNA levels and NSCLC prognosis. Also, evaluation of the impact of combined serum levels of VEGF and Ang-2 in the prognosis of NSCLC and its potential as diagnostic marker of disease.

Material and methods: An unselected cohort of 145 NSCLC cases admitted at Portuguese Institute of Oncology of Porto was recruited to the study. 30 control individuals, from the same geographical area as case subjects, were also recruited. A peripheral blood sample was taken from each individual. mRNA extraction was performed from the blood samples, and measured by the quantitative real-time polymerase chain reaction (qRT-PCR) method. The serum levels of Ang-2 and VEGF of each patient were determined by enzyme-linked immunosorbent assay (ELISA) technique prior to treatment.

Results: There is an association between circulating Ang-2 mRNA levels and OS in all stages of NSCLC and higher levels of Ang-2 mRNA correlate with poorer OS. This relation is more pronounced when considering only patients eligible for antiangiogenic therapies, with stage IV disease. Also, according to the results, circulating Ang-2 mRNA levels independently determine OS in NSCLC patients.

Serum levels of Ang-2 and VEGF are significantly correlated. High serum levels of Ang-2 and VEGF isolated and both combined ($High_{Ang\text{-}2/VEGF}$) correlate with likelihood of presenting NSCLC. Serum levels of Ang-2 and $High_{Ang\text{-}2/VEGF}$, but not VEGF alone, are independent prognostic factors for NSCLC.

Conclusions: This study suggests that circulating Ang-2 mRNA levels could successfully be included as predictive biomarkers of response in the design of clinical trials involving antiangiogenic drugs targeting Ang-2 and that $High_{Ang\text{-}2/VEGF}$ serum levels could be exploited as a new valuable integral biomarker in NSCLC. We hypothesise that in some NSCLC, tumors obviate the need to generate angiogenesis by co-opting host mature vessels and growing along them (vessel co-option). Tumor-co-opted vessel interactions result in endothelial cells (ECs) activation and intense Ang-2 expression and secretion, leading to vascular disruption and vessel regression, generating a hypoxic core in the tumor that is rescued by an increased expression of VEGF, which induces a robust angiogenic response. This gives the rationale for therapeutic approaches of dual inhibition of Ang-2 and VEGF serving as a launchpad to more successful NSCLC anti-vascular treatments.

RESUMO

Introdução: O cancro do pulmão permanece a forma mais incidente e letal de cancro a nível mundial, com uma taxa de mortalidade que representa aproximadamente uma em cada cinco mortes (1.6 milhões de óbitos, no total). Cerca de 85% destes casos correspondem a cancros de pulmão de não-pequenas células (CPNPCs). O cancro do pulmão resulta de um processo que compreende várias etapas, sendo que a angiogénese assume um papel central no desenvolvimento tumoral, permitindo o aporte de oxigénio e nutrientes necessários para o crescimento do tumor. Actualmente, a terapia com inibidores da angiogénese faz parte das guias de tratamento do CPNPC, sendo a via que envolve o Factor de Crescimento do Endotélio Vascular (VEGF) e os seus receptores (VEGFRs) o principal alvo dos inibidores aprovados até à data (bevacizumab e ramucirumab). No entanto, no que concerne à sobrevida global (SG) dos doentes, os benefícios obtidos na prática clínica com estes tratamentos têm sido modestos, já que uma fracção importante dos tumores apresenta resistência intrínseca à terapia e parte dos restantes adquire resistências ao longo do tratamento. Alguns dos mecanismos propostos para justificar estes resultados desapontantes incluem: 1) activação de vias de sinalização pro-angiogénicas alternativas à via do VEGF(R); 2) recrutamento de células pró-angiogénicas para o estroma do tumor e 3) utilização de mecanismos alternativos à angiogénese durante o desenvolvimento tumoral, como a co-opção de vasos pré-existentes. Outro obstáculo ao sucesso das terapias anti-angiogénicas é a ausência de biomarcadores preditivos que possam ajudar a seleccionar doentes para quem estas terapias serão mais eficazes. A angiopoietina-2 (Ang-2), expressa pelas células endoteliais (CEs) é actualmente investigada como um dos mais promissores alvos da terapia anti-angiogénica e parece estar envolvida nos mecanismos de evasão aos inibidores de VEGF anteriormente descritos. Para além disso, a sua expressão é raramente observada nos vasos sanguíneos saudáveis, sendo altamente regulada a nível do ARN mensageiro (ARNm). Estas características particulares tornam a Ang-2 um candidato interessante a biomarcador prognóstico de SG e preditivo nas terapias dirigidas a alvos angiogénicos.

Objectivos: Avaliação da correlação entre os níveis circulantes de ARNm da Ang-2 e o prognóstico de doentes com CPNPC. Avaliação do impacto da combinação de níveis

séricos de Ang-2 e VEGF no prognóstico do CPNPC e do seu potencial como marcador diagnóstico da doença.

Material e métodos: Participaram neste estudo 145 doentes com CPNPC, admitidos no Instituto Português de Oncologia do Porto, e 30 indivíduos saudáveis da mesma área geográfica. Foi colhida uma amostra de sangue periférico de cada doente à data do diagnóstico, a partir da qual se extraiu ARNm, que foi doseado pelo método quantitativo em tempo real da reacção da polimerase em cadeia (qRT-PCR). Os níveis séricos de Ang-2 e VEGF de cada doente foram determinados por um método enzimático (ELISA).

Resultados: Os níveis circulantes elevados de ARNm de Ang-2 estão associados a menor SG em todos os estadios de CPNPC. Esta relação é ainda mais pronunciada quando considerados apenas os doentes com estadio mais avançado (IV) da doença. Também se verificou que níveis circulantes elevados de ARNm de Ang-2 constituem um factor independente de prognóstico da SG.

Existe uma correlação directa entre níveis séricos de Ang-2 e VEGF. Quer isoladamente quer quando combinados, níveis elevados correlacionam-se com a probabilidade de desenvolvimento de CPNPC. Níveis séricos elevados de Ang-2 isoladamente ou combinados com níveis elevados de VEGF ($High_{Ang\text{-}2/VEGF}$), mas não de VEGF isoladamente, são factores de prognóstico para a SG de CPNPC.

Conclusões: Este trabalho sugere que níveis circulantes de ARNm da Ang-2 poderão ser incluídos como biomarcadores preditivos de resposta em ensaios clínicos que envolvam terapias que tenham como alvo a Ang-2. Sugere também que a combinação de níveis elevados de ANG-2 e VEGF no soro possam ser explorados como biomarcadores integrais no tratamento de CPNPC com inibidores da angiogénese. Também fica a sugestão de que, em alguns CPNPCs, o mecanismo de co-opção de vasos pré-existentes do hospedeiro seja a forma preferencial de vascularização do tumor, obviando a necessidade de angiogénese. A interacção entre as células tumorais e os vasos do hospedeiro resulta na activação das CEs destes vasos, com intensa expressão e secreção de Ang-2, conduzindo-os a um estado de dissociação que culmina com a regressão dos vasos e com a geração de uma área de hipoxia central, que despoleta a expressão de VEGF pelas células do estroma, induzindo uma robusta resposta pró-angiogénica. Esta teoria serve de base ao racional para abordagens

terapêuticas de inibição dupla de Ang-2 e VEGF, servindo de catapulta para terapias anti-angiogénicas de maior sucesso clínico.

1. INTRODUCTION

1.1 Overview of lung cancer

Cancer is a major public health issue, representing a leading cause of morbidity and mortality worldwide [1]. In 2013, the global incidence of cancer cases was 14.9 million, with 8.2 million cancer related deaths and, by 2030, an estimated 24 million new cases are expected [1,2]. While advances in diagnostics and treatment have led to a 3.4-fold increase in patient survival over the last 40 years, many disease settings remain with little progress, and with a high rate of recurrence or fatality from metastatic disease [3].

Lung cancer is the most common incident form of cancer worldwide, with an estimated 1.8 million new cases in 2012, with deaths exceeding those from any other type of malignancy, accounting for nearly one in five deaths (1.6 million deaths in total) [4]. Approximately 85% of those cases are currently classified as Non-Small-Cell Lung Cancers (NSCLCs), [5]. The remaining 15% are Small-Cell Lung Cancers (SCLCs), extremely aggressive tumors, morphologically and histologically distinct from NSCLCs and strongly correlated with cigarette smoking [6].

NSCLCs are currently divided in different subsets, according to its histopathological characteristics [7]. The two main NSCLC histological phenotypes are adenocarcinoma (ADC; ±50%), predominantly more peripheral tumours, thought to arise from the alveolar or bronchiolar epithelium (pneumocytes or Clara cells) presenting often glandular histology, and squamous cell carcinoma (SCC; ±40%), which typically arises from the bronchial epithelium of the larger, more central airways [5]. Other subtypes of NSCLC include large cell carcinoma (LCC; ±3%), which is essentially diagnosed by exclusion if tumour cells do not appear glandular or squamous in shape or do not express ADC or SCC biomarkers [5,7] and neuroendocrine (NE) neoplasms of the lung, including typical and atypical carcinoid tumors and excluding SCLC [8].

The vast majority of patients with NSCLC presents at an advanced stage of disease, when curative treatment is no longer a possibility, resulting in a poor prognosis and an overall five year survival around 15.9% [7,9]. This number has only marginally improved during the past few decades, despite the increased understanding and appreciation of the complexity of NSCLC during this period [9].

Current knowledge on the biology of lung cancer shows that it is the result of a multistep process, with intricate combinations of morphological, molecular and

genetic alterations, ultimately leading to a malignant cell agglomerate bearing the phenotypic hallmarks of cancer, defined by Hanahan and Weinberg in 2011 [7,10]. Among these, angiogenesis seems to assume major importance, since gaining access to the host vascular system and the generation of a tumour blood supply to obtain oxygen and nutrients, growth factors and hormones, among others, are rate-limiting steps in tumour progression [11].

1.2 Angiogenesis and cancer

The identification of massive vascularization in tumors dates to 1863 [12] and the importance of tumor angiogenesis has been recognised since 1908 [13], but it was only in the early 1970s, with the work of Folkman, that angiogenesis was acknowledged as a potential target to inhibit cancer progression [14-17]. The therapeutic potential of anti-angiogenic strategies boosted this field, and placed angiogenesis as one of the major areas of cancer research nowadays.

It is now widely accepted that most tumors and metastases originate as small avascular structures which must induce the development of new blood vessels from pre-existing ones, in order to grow beyond a minimum size of 2-3 mm^3 [11,18]. To achieve this, tumors undergo an angiogenic switch, disrupting the equilibrium between pro and anti-angiogenic regulators, favouring pro-angiogenic mechanisms, where signalling molecules induce quiescent endothelial cells (ECs) to continually sprout from existing blood vessels, forming new vessels that help to sustain expanding neoplastic growth [11,19,20], according to the conventional model of angiogenesis, known as angiogenic sprout [21].

Decades of research investigating the molecular basis of angiogenesis led to the discovery of a number of angiogenic molecules that promote tumor angiogenesis [20]. Of all the identified angiogenic pathways, the most critical appears to be the one involving the vascular endothelial growth factor (VEGF) family and its receptors (VEGFR) [22,23].

1.2.1 The VEGF family

The VEGF family consists of five glycoproteins referred to as VEGFA, VEGFB, VEGFC, VEGFD and placental growth factor (PIGF) and distinguishes itself from other angiogenic super families by the largely non-redundant roles of its members [24]. The VEGF ligands bind to and activate three structurally similar type III receptor tyrosine kinases (TKR), designated VEGFR1, VEGFR2 and VEGFR3. The assortment of VEGF ligands has distinctive binding specificities for each of these TKR, with consequent diversity of function [25]. VEGFA and VEGFB have the greatest binding affinity to VEGFR1 and 2, with the majority of angiogenic effects being attributable to VEGFA, the best characterized of the VEGF family members (from now on referred to as VEGF), which is expressed as various isoforms owing to alternative splicing [25]. It stimulates angiogenesis in health and disease by signalling through VEGFR2, whose expression is restricted primarily to the vasculature and is the key mediator of VEGF-induced angiogenesis [25]. VEGFR1 can also bind VEGF, and might function as a decoy receptor that sequesters VEGF from VEGFR2 and negatively regulates angiogenesis, [26], although its precise role in angiogenesis it is still elusive [24]. The role of PIGF in angiogenesis also remains controversial; it exclusively binds to VEGFR1 and it is speculated that it may directly stimulate vessel growth and maturation and recruit proangiogenic bone marrow-derived progenitors and monocyte-macrophage lineage cells [27,28]. The remaining family members, VEGFC and VEGFD, are formed by proteolytic processing (unlike VEGFA, B and PIGF, isoforms originated by alternative splicing), and bind mainly to VEGFR3, appearing to be important contributors to lymphangiogenesis [28].

Ever since the identification of VEGF as the first endothelium-acting specific cytokine in 1983 [18,29,30], its overexpression has been found in several human tumors, including NSCLC [31-35], probably due to its induction under the ischaemic conditions that usually occur at the rim of necrotic and hypoxic regions of the tumor [11]. A growing number of functions of VEGF in the tumor angiogenic process have been unravelled [35]: it triggers multiple signalling networks that enhance ECs proliferation and survival, increasing its migration and invasion capabilities, increases vascular permeability and interstitial pressure of existing vessels and enhances

chemotaxis and mobilization of bone marrow derived endothelial progenitor cells (EPCs) into the peripheral circulation [25,36].

The recognition of the central role of VEGF in tumor angiogenesis turned it into an attractive target for therapeutic intervention in cancer and the VEGF pathway became the main focus of research in the quest for effective targeted anti-angiogenic strategies [29,37]. The extensive investigation in this field has led to the study of several anti-angiogenic agents, including monoclonal antibodies to block VEGF and its receptor VEGFR2 and VEGFR tyrosine kinase inhibitors (TKIs) [38].

1.3 Anti-angiogenic therapy and lung cancer

Presently, there are two anti-angiogenic compounds approved by the American Food and Drug Administration (FDA) for the treatment of NSCLC. Bevacizumab, an anti-VEGF recombinant monoclonal antibody that blocks the binding of VEGF to its high-affinity receptors, was the first angiogenic inhibitor to complete clinical development, showing clinical benefit in patients with metastatic colorectal cancer when combined with chemotherapy [25,38]. It was approved in 2006 for the treatment of advanced non-squamous NSCLC in the first line setting in combination with chemotherapy [35]. Later, in 2014, ramucirumab, a fully humanized monoclonal antibody that targets angiogenesis by specifically binding to VEGFR-2 with higher affinity than its natural ligand VEGF [39], has been approved for the treatment of patients with metastatic NSCLC in second line setting, in combination with docetaxel [40]. Besides these two anti-angiogenic compounds, many others are currently under clinical evaluation, in different stages of clinical trials or waiting for approval for the treatment of metastatic or recurrent NSCLC [41,42].

An important feature that distinguishes the antiangiogenic drugs from other targeted therapies is that these agents are typically given to unselected NSCLC patients for the approved indications [43] and despite the significant clinical achievements of bevacizumab and ramucirumab in different NSCLC treatment settings, the overall survival (OS) benefits from antiangiogenic therapies remain modest [44] and these VEGF pathway inhibitors are failing to produce enduring clinical responses in most patients [21,45]. In a high fraction of them, the tumor is intrinsically refractory to the anti-angiogenic therapy such that disease progression continues ceaseless [46] and

when this is not the case, acquired resistance to therapy can rapidly occur and limit the efficacy of the antiangiogenic treatments [24,47].

1.4 Mechanisms of resistance to antiangiogenic therapy

Tumor resistance to the antiangiogenic therapies (whether intrinsic or acquired), makes the clinical use of VEGF/VEGFR blockers, in patients with advanced NSCLC, more challenging than anticipated by the results of preclinical experiments [24]. The modest success obtained in the clinical practice raised a number of questions, fuelling this already active field of research, to improve anticancer treatment [47,48].

Researchers are aware that the mechanisms underlying tumor resistance to angiogenic inhibitors are complex and diverse, depending upon the location of the tumor, the nature of the tumor itself, the surrounding stroma, the dynamic nature of the angiogenic process, the strong redundancy determined by a continuous cross-interaction between main and alternative pathways and the ability to recruit proangiogenic bone marrow-derived cells to the tumor site, just to mention a few. Until this intricate network underlying tumor resistance is fully understood, and then selectively inhibited, it will probably be difficult to achieve the full efficacy of anti-VEGF(R) and other antiangiogenic therapies [49].

In the last few years, some of the explanations to resistance to current antiangiogenic therapies have been unveiled and include: 1) upregulation of alternative pro-angiogenic signalling circuits to overcome VEGF(R) inhibition; 2) recruitment of vascular progenitor cells and pro-angiogenic monocytes from the bone marrow to tumor stroma, where they promote tumor revascularization and growth and increased capabilities of invasion without angiogenesis [21,23,24,46,47,50]; 3) non-angiogenic tumor microenvironments, as is the case of tumors that do not need angiogenic sprout to obtain an efficient blood supply and rather use alternative vascularization mechanisms to support its growth [46,47,51]. These comprise NSCLCs that grow using pre-existing vessels in the rich vascularized lung, through a vessel co-option strategy [52].

Exhaustive research on this subject reveals that Angiopoietin-2 (Ang-2), a member of the Angiopoietin (Ang) -Tie (type I transmembrane tyrosine kinase receptor) system,

participates or mediates, at least in part, all of the above proposed mechanisms of resistance to angiogenic inhibitors [53].

1.4.1 Angiopoietin-Tie system (Ang-Tie system)

The human Ang-Tie system was identified in the mid-1990s as a family of growth factors essential for blood vessel formation [54]. It consists of two type I transmembrane tyrosine kinase receptors (Tie1 and Tie2) and three secreted ligands, Ang-1, Ang-2 and Ang-4 [53]. Tie1 and Tie2, with its tyrosine kinase domain in the cytoplasm, are preferentially expressed by vascular and lymphatic ECs, although Tie2 expression has been recorded in non-endothelial cells, both in normal tissue and disease, including carcinoma cells and monocytes [55,56]. Unlike Tie1, Tie2 binds directly to angiopoietins and has strong kinase activity [57]. Tie1 is currently considered an orphan receptor, with no known ligand, but has been shown to bind Tie2 and regulate its activity [53,58].

Ang-1 and Ang-2 are the most extensively characterized ligands of Tie2 and their interactions with it comprise an important endothelial cell-specific receptor tyrosine kinase signalling system in angiogenesis [54,55,59,60]. They are secreted glycoproteins [54], which act primarily in the vasculature to control blood vessel development and stability [61] and interact with Tie2 either in a paracrine (Ang-1) or autocrine (Ang-2) manner. Although both Ang-1 and Ang-2 have important roles in angiogenesis, the nature of their contributions is distinct [53].

Studies of loss-of-function have shown that Ang-1-deficient mice present embryonic lethal phenotype, due to aberrant vessel remodelling and maturation, phenocopying the early midgestation death of Tie2-deficient mice, suggesting that Ang-1 is the single, non-redundant, agonist of Tie2 [5,59,62]. Ang-1 is produced primarily by perivascular cells (smooth muscle cells, pericytes), although in the adult, it is found in many types of tissues and is constitutively secreted in low levels throughout the body [63]. Ang-1/Tie2 signaling promotes blood vessel maturation and stabilization [64]; at low (basal) levels, Ang-1 engagement with Tie2 activates downstream signalling, resulting in EC survival signals, in the maintenance of the endothelial barrier, the quiescent state of vasculature and blood vessel assembly, thus ensuring the

resting, anti-thrombotic and anti-adhesive state of the vascular endothelium [53,59,65].

Ang-2 is mainly synthesized by ECs and stored in Weibel-Palade bodies in its cytoplasm, from where it can be rapidly released upon stimulation to act as an autocrine regulator of ECs functions at sites of vascular remodeling [53]. Despite much research in the last decade, the role of Ang-2 in the Ang-Tie signaling axis and in vascular biology in general, is not as straightforward as the one of Ang-1 [57]. Genetic manipulation experiments in mice showed that Ang-2 gain-of-function phenotype resembles Ang-1 deficiency in embryonic development, suggesting that it functions as a natural antagonist of Ang-1 [59,66], but unlike Ang-1, Ang-2 expression is expendable for normal embryonic development, as shown by loss-of-function studies, although Ang-2 deficiency leads to persistent vascular defects after birth [59].

Along with its prominent role in blood vessel biology during vascular remodeling, Ang-2 seems to be an obligate partner of lymphatics maturation. An elegant study performed by Gale and co-workers showed that Ang-2 is not required for the initiation of lymphatic vascular development, but it is absolutely required for their remodeling and normal functioning [67]. Moreover, they found that Ang-2 seemed to substitute for Ang-1 agonistic functions in lymphatics in vivo, since lymphatic dysfunction in Ang-2 deficient mice could be rescued with Ang-1 administration, suggesting that Ang-2 acts as an activating agonist of Tie2 in this situation [62,67].

The dynamic pattern of Ang-2 expression at sites of angiogenesis, such as cyclic vessel regression in ovaries, tumor vascular co-option, and hyaloid vessel regression, supports the concept that the dominant biologic role of Ang-2 is the control of vascular remodeling through the interruption of Tie2 signaling [54,59,62]; Ang-2-Tie2 association allows for destabilization of established blood vessels through the induction of vessel plasticity (e.g. by decreasing pericyte coverage), disrupting the integrity of the blood vessel wall, thereby counteracting vascular normalization, a pre-requisite for sprouting angiogenesis in the presence of other angiogenic molecules or physiologic vascular regression in the absence of such stimuli [68,69]. Moreover, Ang-2 can exert a direct pro-angiogenic Tie2-independent role by directly binding integrins in Tie2 negative ECs, promoting tumor invasion and metastases [61].

1.4.2 Ang-2 and Tie2 expressing-macrophages (TEMs)

Tie2-expressing monocytes/macrophages (TEMs) are a subpopulation of circulating and tumor-infiltrating myeloid cells with inherent vascular growth promoting activity, representing a reservoir of cells innately committed to a proangiogenic function [70,71]. In human tumors, they are found mainly in perivascular and avascular viable areas, but are largely absent in non-neoplastic tissues adjacent to the tumor [72]. The preferential location of TEMs in the vicinity of tumor blood vessels suggests that these cells may cross-talk with ECs and provide paracrine support to nascent blood vessels in these areas during the angiogenic process [72,73]. This notion was reinforced by selective elimination of TEMs using a suicide gene approach, showing that the absence of TEMs from tumors impairs angiogenesis and delays tumor growth, suggesting that these cells have nonredundant, proangiogenic activity in tumors [71,74].

Ang-2 levels in the tumor microenvironment have been directly correlated with increased TEMs recruitment to tumor stroma, and it is well established that Ang-2 is chemoattractant to TEMs, in a process mediated by Tie2 [70,73,75,76]. The overexpression of Ang-2 by ECs and tumor cells exposed to hypoxia, results in greater infiltration of TEMs into tumors [72] and in turn, hypoxia induces upregulation of cell-surface expression of Tie2 in TEMs, increasing their responsiveness to Ang-2, in a mechanism of feed-back loop used to amplify TEMs function in tumor microenvironment [77]. It is described that stimulation with Ang-2 modulates the cytokine profile expressed by TEMs [78], upregulating angiogenic mediators, such as the proangiogenic enzymes thymidine phosphorylase (TP) and cathepsin B (CTSB) and downregulating the expression of antiangiogenic and immunosuppressive cytokines, such as pro-apoptotic tumor necrosis factor-α (TNF-α) and antiangiogenic interleukin-12 (IL-12) [75,77].

Although TEMs exhibit some features of M2-polarized tumor-associated macrophages (TAMs), a few differences have been found between them. TEMs express lower levels of VEGF than TAMs, and do not home to hypoxic, avascular tumor areas. Thus, it is likely that TEMs exert a requisite proangiogenic function by supporting tumor angiogenesis downstream to VEGF-induced vascular activation, regulating blood vessel formation by a VEGF independent pathway [76]. This could explain why the

presence of TEMs in the tumors counteracts the efficacy of antivascular treatments [73], promoting vascular regrowth following therapy-induced vascular damage and its association with the heightened invasive phenotype observed upon the use of anti-angiogenesis therapies [79].

1.4.3 Ang-2 involvement in alternative pathways of tumor vascularization

It is fairly recognized that tumor progression is heavily dependent upon angiogenesis. However, the model that angiogenesis is necessary for a tumor to become larger than a few millimetres and become clinically detectable has been challenged by the extensive research in this field, which has shown that angiogenesis is not always a pre-requisite for tumor growth [80]. When tumors arise in well-vascularized organs, their growth will rely on the invasion of host tissue. Enhancement of invasion and metastasis facilitates access to normal tissue vasculature, and cancer cells stay in close contact with the surface of blood vessels [42,81,82]. This allows tumor cells to grow and migrate along quiescent normal vessels and take their oxygen and essential nutrients without obligate neovascularization, in a process known as vessel co-option [46,47,83]. This procedure has been recognized as an important mechanism to establish tumor vasculature, especially in more aggressive tumors, and represents a major route for a solid tumor to evade antiangiogenic therapy [45,46,51,84].

The co-opted vessels are usually supported by pericytes, which stabilize it, while promoting endothelial cell survival via induction of autocrine VEGF signalling [85]. In vessel co-option, the first tumor-vessel interactions result in ECs activation and intense Ang-2 expression and secretion [61]; Ang-2 then acts through an endogenous autocrine loop mechanism that is context dependent [65,86]. When it binds to its Tie2 receptor, it functions as a vessel-destabilizing molecule that converts mature vessels to a tenuous and plastic state by inducing loosening of endothelial cell interactions with pericytes and smooth muscle cells, leading to the loss of vascular integrity and increased vascular permeability that facilitates the infiltration of proteases, cytokines and angiogenic myeloid cells, and thus, the priming of the vasculature for a robust angiogenic response in the presence of growth factors, such as VEGF [18,59,64,65,76,87,88].

In the absence of VEGF, as during the early phases of antiangiogenic treatment, the expression of Ang-2 causes irreversible loss of vascular structures [87,89] with marked regression of the co-opted vessels [53] in a very similar fashion to what happens with primitive vessels during development [86]. This generates the hypoxic core and the apoptotic tumor cell loss observed in nonangiogenic tumors [84,87], that presumably act as de initial stimulus for the molecular changes (which are yet to be clarified), that culminate in VEGF expression by the remaining tumor cells and in neoangiogenesis [90], mediated both by VEGF and Ang-2 [84].

1.4.4 VEGF and Ang-2-Tie2 axis inhibition

Ang-2 seems to be a preferential partner of VEGF in the orchestration of tumor angiogenesis. Similarly to what is seen with VEGF expression, elevated levels of Ang-2 have been associated with advanced disease, progression and poor prognosis in the most diverse tumor models, including NSCLC [64,90], glioblastoma, gastric, colorectal, breast, prostate, kidney and hepatocellular carcinomas, as well as multiple myeloma, melanoma, and neuroendocrine tumors [91-99], but unlike VEGF, Ang-2 is seldom detectable in healthy vasculature, making it the perfect target to tumor therapy [62].

Considering the multifaceted nature of Ang-2 in tumor angiogenesis promotion and its proposed involvement in the mechanisms of resistance to VEGF targeted therapies, it is not surprising that, nowadays, Ang-2/Tie2 system is regarded as one of the most important therapeutic targets in antiangiogenic strategies. Its inhibition would allow both the optimization of current antiangiogenic strategies and the circumscription of acquired resistance to approved angiogenic inhibitors [53,64,69,76,87,100-107]. Targeting Ang-2/Tie2 axis and VEGF(R) pathway holds the promise of more clinically meaningful responses than monotherapies targeting VEGF pathway alone [27,53].

1.5 Biomarkers and antiangiogenic therapy

The missing key to optimize the results obtained with angiogenic inhibitors would be the distinction between patients that are likely to respond to these treatments from those intrinsically refractory to angiogenic therapies. Predictive biomarkers are greatly needed to achieve this goal [108-110]. Despite the several molecular mediators

of angiogenesis and inflammatory signalling that have been investigated as potential biomarkers of antiangiogenic therapy in NSCLC, no biomarker has yet been prospectively validated to correlate with outcomes [111-113].

In the future, it is essential that the selection of new antiangiogenic agents to enter early pre-clinical trials is based on the availability of identified and validated biomarkers predictive of future clinical efficacy [44,109,114].

2. OBJECTIVES

2.1 General objectives

- Evaluate the correlation between circulating Ang-2 mRNA levels and NSCLC prognosis in an unselected cohort of NSCLC patients.

- Evaluate the prognostic relevance of serum Ang-2 and VEGF levels in an unselected cohort of NSCLC patients.

2.2 Specific objectives

The aims of this study include:

- Determination of the independent prognostic value of circulating Ang-2 mRNA levels in OS of NSCLC patients, by a quantitative real-time polymerase chain reaction (qRT-PCR) method.

- Investigation of the correlation between serum Ang-2 and VEGF levels in NSCLC patients by enzyme-linked immunosorbent assay (ELISA) technique.

- Assessment of the impact of the combined serum Ang-2/VEGF levels in the prognostic of the disease.

- Evaluation of its potential value as a diagnostic cancer marker

- Assessment of the influence of the combined serum Ang-2/VEGF levels in the susceptibility to NSCLC.

- Help to establish candidate markers for better prognostication for NSCLC patients

3. RESULTS / PUBLICATIONS

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Circulating Ang-2 mRNA Expression Levels: Looking Ahead to a New Prognostic Factor for NSCLC

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Abstract

Non-small cell lung cancer (NSCLC) is the most common cancer and the leading cause of death from cancer worldwide. Antiangiogenic strategies directed towards tumor stroma are becoming gold standard in NSCLC treatment and researchers have been searching for biomarkers to identify patients for whom therapy with antiangiogenic inhibitors may be most beneficial and the importance of these as prognostic factors in NSCLC. The purpose of this study was to evaluate the prognostic value of circulating Ang-2 mRNA levels prior to treatment in NSCLC patients. The mRNA levels were determined by quantitative real-time PCR in the peripheral blood of 92 NSCLC patients. Our results demonstrate that patients with high circulating Ang-2 mRNA levels have diminished overall survival when compared to those with low mRNA levels (20.3 months vs 34.3 months, respectively; Log Rank Test, $p = 0.016$), when considering all NSCLC stages and this difference is even bigger when considering only patients with stage IV (15.9 months vs 31.3 months, respectively; Log Rank Test, $p = 0.036$). Moreover, circulating Ang-2 mRNA levels independently determine overall survival, and the concordance (c) index analysis showed that the definition of a nomogram that contains information regarding tumor stage, patients' smoking status and circulating Ang-2 mRNA levels present an increased capacity to predict overall survival in NSCLC patients (c-index 0.798). These results suggest that this nomogram could serve as a unique and practical tool to determine prognosis in NSCLC, not relying on the availability of adequate surgical or biopsy specimens of NSCLC. Attending to our results, the circulating Ang-2 mRNA levels should also be included in the design of preclinical studies and clinical trials involving antiangiogenic drugs targeting Ang-2, to guide adequate patient stratification and dose selection and increasing the likelihood of benefit to a level that is acceptable to patients and clinicians.

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Introduction

Non-small-cell lung cancer is the most frequent type of lung cancer and the most common cause of death from cancer [1]. In 2010, the number of deaths from lung cancer worldwide was 1·5 million, representing 19% of all cancer deaths that year. Most lung cancers (~80%) are non-small-cell lung cancers (NSCLC) and of these patients, more than 65% present with locally advanced or metastatic disease [2].

Solid tumors, including NSCLC, require angiogenesis—the formation of new blood vessels from existing vessels—for survival, growth, and metastasis. These new tumor vessels are structurally and functionally abnormal. They develop by sprouting or intussusception from pre-existing vessels and exist in a constantly dynamic state of sprout formation, proliferation, remodeling, or regression [3,4].

In the last 9 years, antiangiogenic therapy has become part of standard antitumor treatment. However, the clinical efficacy of such therapies is limited, and it appears that the full therapeutic potential of antiangiogenic intervention has not been fully exploited [5].

It's now known that there are various molecular players involved in different mechanisms of vascular growth in solid tumors, and among these, members of the Vascular Endothelial Growth Factor (VEGF) and Angiopoietin (Ang) family have a predominant role [3].

Angiopoietins, the bona fide ligands of Tie-2 receptor, form a family of secreted 70 kDa glycoproteins acting primarily on the vasculature to control blood vessel development and stability. Four distinct angiopoietins have been described: Ang-1, Ang-2, Ang-3 and Ang-4. Angiopoietins bind the second immunoglobulin motif of Tie-2 whereby they activate Tie-2 and, indirectly, Tie-1 in Tie-1/Tie-2 heterodimers [6].

Ang-1 is expressed by pericytes, smooth muscle cells, and fibroblasts and acts in a paracrine manner. In contrast, Ang-2 is expressed by endothelial cells (EC) and stored in the Weibel-Palade bodies from where it can be rapidly released on stimulation to act as an autocrine regulator of EC functions [7].

Ang-1 and Ang-2 have been described to exert opposing functions during vessel development. Ang-1–induced Tie2 activation transduces survival signals and leads to vessel stabilization and maturation. In turn, Ang-2 acts as a vessel destabilizing agent that induces permeability and leads to dissociation of cell-cell contacts in cultured endothelial cells. Genetic experiments have solidly established Ang-2 as an antagonistic Tie2 ligand [7]. Moreover, Ang-2 can have a direct pro-angiogenic Tie-2-independent role by directly binding integrins in Tie2 negative EC [6].

Ang-2 has been implicated in the remodeling of the tumor vasculature in a process resembling its physiological actions [8,9]. Among the first steps of the angiogenic switch is the co-optive engagement of the pre-existing host vasculature by the growing tumor. This results in EC activation and intense Ang-2 expression, which promotes the dissociation of pericytes from pre-existing vessels and increases vascular permeability, which facilitates the infiltration of proteases, cytokines and angiogenic myeloid cells, and thus, the priming of the vasculature for a robust angiogenic response in the presence of growth factors, such as VEGF-A [6]. Following the angiogenic switch, the Ang–Tie system contributes, in concert with VEGF, to tumor angiogenesis [10].

Ang2 is strongly regulated at the transcriptional level. In fact, almost any form of endothelial cell activation leads to upregulation of Ang2 mRNA. The mRNA induction of Ang2 in tumor endothelium has made Ang2 a very attractive circulating biomarker of angiogenic activation [5].

Some studies have addressed the correlation between Ang-2 expression in tumor tissue and the protein circulating levels and cancer development and metastasis. However, few clinical studies have documented a correlation between this molecule and disease clinical features or prognosis in lung cancer [11-15].

To the best of our knowledge, the present study is the first to establish a correlation between circulating Ang-2 mRNA levels and lung cancer prognosis.

Materials and Methods

Ethics statement

The study was conducted according to the principles of the Helsinki Declaration. The study was approved by the local ethics committee at the Portuguese Institute of Oncology of Porto (Portugal). All individuals signed a written informed consent prior to the inclusion in the study.

Study Population

The study included Caucasian patients from the North region of Portugal. The inclusion criteria were histological or cytological confirmed diagnosis of NSCLC, no previous treatment, an Eastern Cooperative Oncology Group (ECOG) performance status$\leq$2 (with 0 indicating that the patient is fully active, 1 that the patient is ambulatory but restricted in strenuous activity, and 2 that the patient is ambulatory and capable of self-care but is unable to work [16]), no prior oncologic disease and available clinical data.

Circulating mRNA levels quantification

Circulating Ang-2 mRNA levels were analyzed by quantitative real-time PCR (qRT-PCR). Initially, the mRNA was isolated from the cell fraction of peripheral blood samples by TriPure reagent (Roche Applied Science), and after separation of the RNA fraction, the samples were purified using the commercial kit GeneJET RNA Purification Kit (Fermentas). RNA samples were then used as templates for cDNA synthesis, using a High Capacity RNA-to-cDNA Kit (Applied Biosystems), according to the manufacturer's instruction. Finally, qRT-PCR was carried out on a StepOne ^{TM}One qPCR equipment, containing 1x Master Mix (Applied Biosystems), with 1x probe (TaqMan Gene Expression Assay with reference number Hs 01048042_m1, Applied Biosystems), cDNA sample, human GUSB (Beta Glucuronidase) and human β-2M (β-2 Microglobulin) endogenous controls (both from Applied Biosystems) according to manufacturer's instructions.

To quantify the amplified transcripts, we used the comparative CT ($2^{-\Delta\Delta C}$T) method [17]. In accordance with the method, the mRNA amounts of the target gene (*Ang-2*) were normalized to two endogenous controls and relatively to a calibrator. We used the housekeeping genes *GUSB* (Applied Biosystems) and *β-2M* (Applied Biosystems) as internal controls and commercial RNA controls as calibrators (Applied Biosystems). ΔΔCT represents the difference between the mean ΔCT value of a patient blood sample and the mean ΔCT value of the calibrator, both calculated after the same PCR run, whereas ΔCT is the difference between the CT of the target gene and the CT of the endogenous reference gene of the same sample. The relative quantitative value was expressed as $2^{-\Delta\Delta}$CT. Relative quantification (RQ) based on the Ct (the number of PCR cycles necessary to obtain the threshold signal of fluorescence) values was analyzed using Applied Biosystems StepOne Software v 2.2. All samples were run in duplicate.

Statistical analysis

Ang-2 mRNA expression levels were considered as categorical variables using the first quartile as cut-off point. We defined that the values under the cut-off point should be included in the low expression group and that all the other cases above the cut-off point constituted the high expression group. Overall Survival (OS) was calculated from the beginning of treatment to death from any cause. Median OS was estimated with the Kaplan-Meier method and compared with a two-sided log-rank test. Multivariate Cox proportion analysis was performed to determine the influence of age, gender, tumor stage, histological type, smoking status and circulating Ang-2 mRNA levels on OS in NSCLC patients. Hazard ratios (HRs) estimated from the Cox analysis were reported as relative risks with corresponding 95% confidence intervals (CIs). The extent of discrimination of the predictive ability was quantified using the Harrel's concordance index (c-index), which estimates the probability of concordance between predicted and observed responses. The interpretation of the C index is similar to the interpretation of the area under a receiver operating curve (ROC) curve. A value of 1.0 indicates that the features of the model perfectly separate patients with different outcomes while a value of 0.5 indicates the features contain prognostic information equal to that obtained by chance alone.

All analyses were performed using Statistical Package for Social Science (SPSS) for Windows version 18 (Chicago, IL). The level of statistical significance was set at 5% ($P\leq0.05$).

Results

The study included 92 Caucasian individuals from the North region of Portugal, with histopathological diagnosis of NSCLC, with a mean age of 63.2 years$\pm$10.7. The blood samples were collected at the time of diagnosis, before treatment, and included 33 squamous cell carcinomas (SCC), 46 adenocarcinomas, 11

Table 1. NSCLC patients' characteristics.

	Patients (n = 92)	
	n	%
Gender		
Female	21	22.8
Male	71	77.2
Age		
Mean±S.D.	63.2±10.7	
Histology		
Adenocarcinoma	46	50.0
SCC	33	35.8
NSCLC NOS	11	12.0
Large Cells	1	1.10
Mixed	1	1.10
Staging		
I	3	3.30
II	1	1.10
III	38	41.3
IV	50	54.3
Smoking status		
Non-smoker	22	23.9
Smoker	48	52.2
Former smoker	22	23.9

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undifferentiated NSCLC, 1 large cells and 1 mixed carcinomas, of which 77,0% were male and 75,8% smokers or former smokers, divided in 42 non-metastatic and 50 metastatic cases (Table 1).

Our results demonstrate that patients with high circulating Ang-2 mRNA levels have diminished overall survival when compared to those with low mRNA expression (20.3 months vs 34.3 months, respectively; Log Rank Test, $p = 0.016$) (Figure 1). Moreover, when considering only stage IV patients, the most suitable candidates to antiangiogenic treatment, the range interval between overall survival in the high and low settings of Ang-2 mRNA expression augments (15.9 months vs 31.3 months, respectively; Log Rank Test, $p = 0.036$) (Figure 2).

To determine the independent prognostic value of circulating Ang-2 mRNA levels for OS, a multivariate analysis using a Cox proportional hazard model was performed. In the multivariate analysis that included age, gender, tumor stage, histological type, smoking status and circulating Ang-2 mRNA levels, we identified tumor stage, smoking status and Ang-2 mRNA levels as independent prognostic factors for OS in NSCLC patients (Table 2).

Attending to these results, we performed an analysis considering four different prognostic models to ascertain the predictive power of circulating Ang-2 mRNA levels in the clinical outcome of NSCLC patients (Table 3). In the first two models, we considered the predictive ability of tumor stage and smoking status (c-index 0.657 for tumor stage and 0.522 for smoking status) (Model 1 and Model 2). Model 3 addressed the question whether circulating Ang-2 mRNA levels could also be considered a prognostic factor in NSCLC, with a c-index of 0.629 (Model 3). In model 4, we created a three variables prognostic nomogram congregating the three aforementioned models. The prognostic predictive ability was increased when adding circulating Ang-2 mRNA levels, with a c-index of 0.798 (Model 4).

Discussion

Many predictive and prognostic markers have been assessed in NSCLC but, until the discovery of the importance of Epidermal Growth Factor Receptor gene (*EGFR*) [18], no single molecular marker had proven to be useful for either patient selection or selection of specific drugs. The TNM classification (lung cancer staging) has stood the test of time and to date, no other prognostic factors beyond it have been prospectively validated, remaining the most powerful prognostic instrument in lung cancer [19,20]. Hence, the identification of other prognostic factors that can be integrated with TNM to create a composite prognostic index for NSCLC would be clinically useful.

Many clinical trials have demonstrated the importance of evaluating several molecular biomarkers of NSCLC tumor specimens to allow a personalized medicine, enhancing progression free survival and overall survival times, diminishing side effects, giving patients a better quality of life and enabling to perform more cost-effectiveness treatments [21]. Moreover, we now know that in addition to predictors of response, these genes can also be regarded as prognostic factors for NSCLC [21], making the evaluation of these biomarkers the state of the art of the advanced or metastatic NSCLC treatment. However, only patients with lung adenocarcinoma with *EGFR* mutations or anaplastic lymphoma kinase (*ALK*) rearrangements have an FDA-approved therapy available [18,21–23]. Unfortunately, for squamous cell NSCLC the scenario is even more shadowy. Although this is an important field where novel targeted therapies are currently under investigation, the disease prognosis still remains disappointing.

As the field of lung cancer moves further into the age of personalized medicine, alternative targets continue to be investigated, since it has become clear that it will be imperative to target the tumor stroma and surrounding environment and not merely the genes' mutations within the cancer cells itself. The main goal of this quest is to identify a pan-NSCLC prognostic factor that can also help to predict treatment response and monitor tumor progression. One of the most extensive studied of such alternative targets is angiogenesis, a necessary process in the growth and metastasis of all solid tumors [23]. Preclinical models and selected clinical trials showed benefits for targeting angiogenesis in lung cancer, with antiangiogenic treatment emerging as the first effective anti-stroma therapy to complement the established antitumor therapies [7]. So far, only VEGF, the master switch of the angiogenic cascade, has been validated as a therapeutic target for antiangiogenic intervention [7] but no published clinical study has proved that circulating levels of this target are prognostic factors in patients with NSCLC subjected to antiangiogenic therapy [24].

Despite of the advances into the elucidation of the tumor milieu in general and tumor angiogenesis in particular, there is a significant knowledge deficit in the understanding of the molecular basis of antiangiogenic therapy and the related adverse events seen with these agents [23]. Researchers have been searching for potential biomarkers to identify patients for whom therapy with antiangiogenic inhibitors may be most beneficial and the importance of these as prognostic factors in NSCLC.

Whereas VEGF is abundantly expressed by the tumor cells in most tumors, Ang-2 is mostly expressed by the tumor-associated endothelium [7]. Moreover, unlike VEGF, Ang-2 shows limited

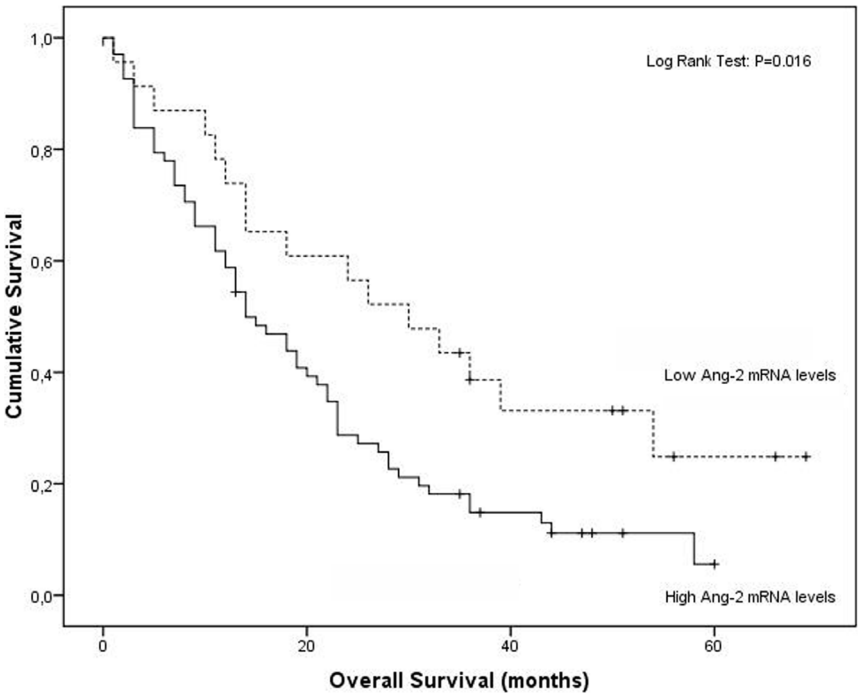

Figure 1. Association of high and low circulating levels of Ang-2 mRNA with overall survival in NSCLC by Kaplan-Meier curves.
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postnatal expression in normal tissues and its broad expression and prominent upregulation in tumor milieu turns it in the perfect candidate to help to define prognosis in solid tumors, besides being a suitable suspect in the game of antiangiogenic strategies [7,25].

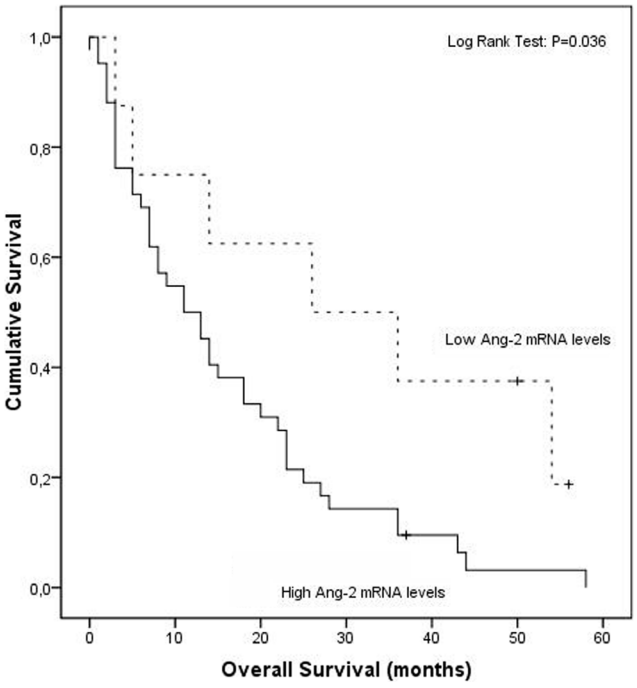

Figure 2. Association of high and low circulating levels of Ang-2 mRNA with overall survival in stage IV NSCLC by Kaplan-Meier curves.
doi:10.1371/journal.pone.0090009.g002

In the present study, we aimed to evaluate the prognostic significance of Ang-2 mRNA detection in the cell fraction of peripheral blood of patients with NSCLC prior to treatment, using qRT-PCR. Moreover, we wanted to assess the possibility of using it as a prognostic factor that could be adjoined to NSCLC staging to create a composite prognostic index for NSCLC and created a nomogram that predicts the influence of circulating Ang-2 mRNA levels in NSCLC clinical outcome.

Our results demonstrate that high circulating Ang-2 mRNA levels are a significantly unfavorable prognostic factor in NSCLC overall survival. Patients with high circulating Ang-2 mRNA levels have diminished overall survival when compared to those with low mRNA expression, when considering all NSCLC stages and this difference is even bigger when considering only patients with distant metastasis, the most suitable candidates to antiangiogenic therapies. Moreover, mRNA levels independently determine survival, and its prognostic predictive ability increases when modeled in a simple and easy to apply nomogram with NSCLC staging, patients' smoking status and Ang-2 mRNA levels (c-index 657 vs c-index 0.798, respectively).

Table 2. Multivariate Cox regression analysis for predictable factors of overall survival.

	HR	95% CI	P
Age (≥63;<63)	0.97	0.0.610–1.55	0.905
Gender	0.55	0.260–1.16	0.118
Tumor stage	1.79	1.12–2.88	0.016
Histological type	1.12	0.810–1.53	0.503
Smoking status	2.36	1.09–5.09	0.029
Ang-2 mRNA	2.04	1.13–3.67	0.017

doi:10.1371/journal.pone.0090009.t002

Table 3. Predictive models of OS according to independent prognostic factors.

	HR	95% CI	P	c-index
Model 1				
Tumor stage	2.30	1.85–2.86	<0.0001	0.657
Model 2				
Smoking status	1.15	0.90–1.47	0.266	0.522
Model 3				
Ang-2 mRNA	1.92	1.10–3.36	0.021	0.629
Model 4 - Nomogram				0.798
Tumor stage	1.81	1.13–2.90	0.013	
Smoking status	1.58	0.91–2.74	0.103	
Ang-2 mRNA	1.94	1.09–3.43	0.024	

doi:10.1371/journal.pone.0090009.t003

Taken together, these results prompt us to think that detection and quantification of circulating Ang-2 mRNA in blood samples, along with proper NSCLC staging, could serve as a unique and practical diagnostic tool to determine prognosis in NSCLC. Circulating Ang-2 mRNA levels samples are a simple to obtain factor which can theoretically reflect the overall angiogenic activity of the tumor and offers a huge advantage over tissue based markers, including the ability to carry out continuous, noninvasive assessments over time and most important, not relying on the availability of adequate surgical or biopsy specimens of NSCLC.

Various therapeutic agents targeting Ang-2 have been described and are being evaluated in early-phase clinical trials [5,9,25–28]. Albeit antiangiogenic drugs are efficacious in unselected populations, increasing market competition between targeted therapies is likely to drive the growth of individualized chemotherapy, with a central role for biomarkers. Although more studies are needed to confirm this hypothesis, the circulating Ang-2 mRNA levels could be strong candidates for predicting the survival benefit associated with the targeted therapies currently under evaluation and should be included in the design of preclinical studies and clinical trials involving antiangiogenic drugs targeting Ang-2, to guide adequate patient stratification and dose selection and increasing the likelihood of benefit to a level that is acceptable to patients and clinicians.

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Author Contributions

Conceived and designed the experiments: ALC AA MG. Performed the experiments: ALC MG. Analyzed the data: ALC RC RM. Contributed reagents/materials/analysis tools: ALC AA AM RM. Wrote the paper: ALC AA AM RM.

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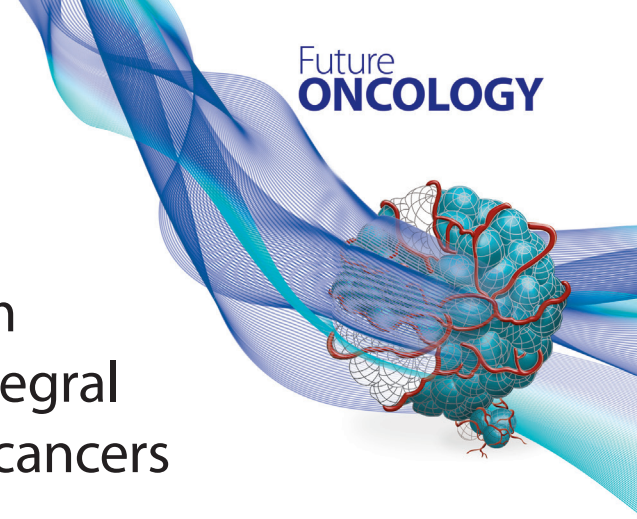

Combined Ang-2 and VEGF serum levels: holding hands as a new integral biomarker in non-small-cell lung cancers

Ana Luísa Coelho[1,2], António Manuel Araújo*[,3,4], Mónica Patrícia Gomes[1,4], Raquel Jorge Catarino[1,4], Elva Bonifácio Andrade[5], Agostinho Marques Lopes[2,6] & Rui Manuel Medeiros[1,4,7]

Aim: Evaluate if serum levels of VEGF and Ang-2 are correlated in non-small-cell lung cancers (NSCLCs) and its implications in the diagnostic and prognostic of the disease. **Patients & methods:** Unselected cohort of 145 NSCLC patients and 30 control individuals. The serum levels of Ang-2 and VEGF of each patient were measured by ELISA prior to treatment. **Results & conclusions:** Serum levels of Ang-2 and VEGF are correlated ($p < 0.0001$). High serum levels of Ang-2 and VEGF isolated and both combined ($high_{Ang\text{-}2/VEGF}$) correlate with likelihood of presenting NSCLC ($p = 0.016$; $p = 0.003$; $p < 0.0001$, respectively). Serum levels of Ang-2 and $high_{Ang\text{-}2/VEGF}$ but not VEGF alone are independent prognostic factors ($p = 0.001$; $p = 0.619$; $p = 0.005$). $High_{Ang\text{-}2/VEGF}$ serum levels could be exploited as a new valuable integral biomarker in NSCLC.

KEYWORDS
• Ang-2 • antiangiogenic therapies • biomarkers • NSCLC • VEGF

Lung cancer is one of the leading causes of death from cancer worldwide. In 2008, it was the most commonly diagnosed cancer as well as the principal cause of cancer death in males globally and it was the fourth most commonly diagnosed cancer and the second cause of cancer death in females [1]. Most lung cancers (~80%) are non-small-cell lung cancers (NSCLCs) and of these patients, more than 65% present with locally advanced or metastatic disease [2].

The advent of targeted agents that inhibit tumor specific biological pathways opened the door for a new era of NSCLC treatment. However, only *EGFR* mutations (~10–15% in the occidental population) or anaplastic lymphoma kinase rearrangements (~3–5% in the occident) have a US FDA-approved therapy available [3]. Alternative targets continue to be investigated and one of the targeted approaches most widely studied in the treatment of NSCLC is the inhibition of angiogenesis [4].

Abnormal angiogenesis, characterized by an increase in the number of proliferating endothelial cells and altered morphology of the vasculature, is a hallmark of cancer [5]. VEGF has long been regarded as the master switch of angiogenesis induction, orchestrating early events of the angiogenic cascade, such as directional sprouting and endothelial cell proliferation [6–8]. There is also a wealth of evidence supporting the role of VEGF as a potent regulator of solid tumor angiogenesis, including NSCLC: VEGF is strongly expressed in tumors from patients with NSCLC and correlates strongly with microvessel density, survival and postoperative relapse [9]. Despite its requisite to vascular formation, VEGF needs to work in concert with other factors and several studies suggest that the angiopoietins are among its preferential partners [6,10].

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Ang-1 and Ang-2 are secreted factors that bind to the endothelial cell-specific receptor tyrosine kinase Tie2 [11]. Angiopoietin/Tie2 signaling system is essential for vascular development and function [12]. Ang-1 is a strong Tie2 agonist that is produced primarily by perivascular cells, and Ang-1/Tie2 signaling is believed to promote blood vessel maturation and stabilization [11]. By contrast, Ang-2 expression is tightly regulated, and it is synthesized and secreted primarily by endothelial cells (EC) at sites of vascular remodeling and is believed to function largely as a Tie2 inhibitor, blocking Ang-1 function, disrupting the integrity of the blood vessel wall and inducing a state of vascular plasticity, thereby counteracting vascular normalization, which conducts to angiogenesis [13,14].

Insights into tumor angiogenesis have shown that VEGF and Ang-2 act in concert during tumor vascular development [10,15]. Following the angiogenic switch when proangiogenic stimuli for new vessel formation become dominant over naturally occurring angiogenesis inhibitors and tumor changes from an avascular state to an angiogenic phenotype [8,16], there is the activation of EC and intense Ang-2 expression, which leads to EC apoptosis and the regression of co-opted blood vessels. This secondary avascular tumor experiences profound hypoxia, which upregulates VEGF expression to induce robust angiogenesis at the tumor margin. In the presence of VEGF, Ang-2 enables EC migration, proliferation and sprouting of new vessels [12].

Many biomarkers of angiogenesis have been proposed and investigated as diagnostic and prognostic markers in cancer, but despite extensive research, none has yet been validated for routine clinical use [9,17].

As agents targeting Ang-2, alone or in combination with VEGF inhibition, are currently in Phase I and Phase II trials [18,19], we aimed to evaluate the prognostic relevance of serum Ang-2 and VEGF levels in an unselected cohort of NSCLC patients. Based on the proposed interplay between VEGF and Ang-2, it was also examined if there was a co-dependent relation in both molecules serum levels and whether the combination of both had a diagnostic and/or prognostic impact in NSCLC patients or in the susceptibility for NSCLC development.

Patients & methods

• Study population

The study included 145 newly diagnosed and untreated Caucasian NSCLC patients, from the north region of Portugal, admitted to the Portuguese Institute of Oncology of Porto, and a control group with 30 healthy individuals from the same geographic area. Patients' recruitment started in 2006 and the inclusion criteria were histological or cytological confirmed diagnosis of NSCLC, no previous treatment, an Eastern Cooperative Oncology Group (ECOG) performance status ≤2 (with 0 indicating that the patient is fully active, 1 that the patient is ambulatory but restricted in strenuous activity and 2 that the patient is ambulatory and capable of self-care but is unable to work) [20], no prior oncologic disease and available clinical data. None of the patients performed antiangiogenic therapy. All the patients were chemonaive by the time of sampling collection and performed chemotherapy as first-line treatment or in combination with surgery. The patients included in this study were treated according to the guidelines at the time of the study, namely in first line with a platin-based doublet chemotherapy in combination with a third-generation cytotoxic compound such as paclitaxel, gemcitabine or pemetrexed. In second and posterior lines, they were treated with docetaxel, pemetrexed or erlotinib.

The control group consisted of presumably Caucasian healthy individuals, recruited from the blood donor bank of the Portuguese Institute of Oncology of Porto, free of malign, inflammatory and connective tissue diseases.

• Serum Ang-2 & VEGF analysis

All study participants had venous blood samples drawn before any treatment. Blood samples were collected in CTAD tubes to avoid platelet and leukocyte secretion of VEFG [21]. All the samples were collected in the morning, after an overnight fasting and allowed to rest for 1 h, at room temperature, before processing. These were centrifuged at 1000× g for 10 min followed by serum collection. Serums were aliquoted and stored at -20°C until analyses were performed. The scientists were blinded to the outcome of the individual patient.

Serum levels of VEGF and Ang-2 were measured by means of an ELISA using the Quantikine Human VEGF ELISA kit (catalog #DVE00) and the Human Angiopoietin-2 Quantikine ELISA kit (catalog #DANG20), respectively (both were purchased from R&D Systems, Inc., MN, USA), according to manufacturer's protocol. Optical density was measured as the end point for the

concentration in each well. Standard curves for each analyte were generated in duplicate on the same plates as the specimens and used to extrapolate the concentrations of the specific analytes using the average value for each sample after subtracting the background absorbance. All specimens were assayed twice and the average of the two measurements was used in the data analysis. The minimum detectable levels of Ang-2 and VEGF were 1.2 and 9.0 pg/ml, respectively.

• Statistical analysis

The Spearman correlation coefficient test was applied to assess the relationship between Ang-2 and VEGF serum levels. These levels were considered as categorical variables using the mean values obtained to the control group as cut-off points.

Chi-square analysis was used to compare categorical variables and a 5% level of significance was used in the analysis. The odds ratio (OR) and its 95% CI were calculated as a measurement of the association between the amount of Ang-2 and VEGF and the probability of lung cancer presence. Multivariate logistic regression analysis was used to calculate the adjusted OR (aOR) and 95% CI, with adjustment for age, gender and smoking status.

For the diagnostic discrimination of serum Ang-2 and VEGF between cancer patients and controls, the area under the curve of the receiver operating characteristic curve (AUC–ROC) was assessed nonparametrically. A p-value ≤ 0.05 was regarded as significant. An AUC-ROC equal to 1 denotes perfect discrimination between patients with cancer and patients without cancer, a value equal to 0.5 denotes the lack of discrimination, and values in between indicate a degree of discrimination between strong and poor.

Overall survival (OS) was calculated from the beginning of treatment to death from any cause. Median OS was estimated with the Kaplan–Meier method and compared with a two-sided log-rank test. Multivariate Cox proportional hazard regression was performed to evaluate Ang-2 and VEGF serum levels and NSCLC survival while adjusting for age, gender, smoking history, histological type and clinical stage. Hazard ratios (HRs) estimated from the Cox analysis were reported as relative risks with corresponding 95% CIs.

The predictive discriminative ability of regression models was quantified using the Harrel's concordance index (c-index). C-index estimates the probability of concordance between predicted and observed results and its interpretation is similar to the one of the area under a receiver operating curve (ROC) curve. A value of 1.0 indicates that the features of the model perfectly separate patients with different outcomes while a value of 0.5 indicates that the features contain prognostic information equal to that obtained by chance alone.

All analyses were performed using Statistical Package for Social Science for Windows version 18 (Chicago, IL, USA). The level of statistical significance was set at 5% ($p \leq 0.05$).

Results

Serum samples were collected from 145 NSCLC patients enrolled in this study (prior to treatment, at the time of diagnosis) and 30 control individuals, from March 2006 until September 2011. The median follow-up duration was 22 months (range: 1–63 months).

Table 1 summarizes the baseline characteristics of the lung cancer patients and the controls included in this study.

• Serum Ang-2 & VEGF in NSCLC patients & healthy control individuals

Median serum level of Ang-2 in NSCLC patients was 3432.5 pg/ml (values ranged from

Table 1. Baseline characteristics of non-small-cell lung cancers patients and controls.

Characteristics	Patients (n = 145), n (%)	Controls (n = 30) , n (%)
Gender:		
– Female	33 (22.8)	6 (20.0)
– Male	112 (77.2)	24 (80.0)
Smoking status:		
– Smokers/former smokers	105 (72.4)	24 (80.0)
– Nonsmokers	36 (24.8)	6 (20.0)
– Not stated	4 (2.80)	
Age (years):		
– Mean ± standard deviation	63.8 ± 10.3	58.7 ± 4.63
Histology:		
– Adenocarcinoma	72 (49.6)	
– SCC	51 (35.2)	
– NSCLC NOS	19 (13.1)	
– Large cells	2 (1.4)	
– Mixed	1 (0.7)	
Staging:		
– I	6 (4.1)	
– II	3 (2.1)	
– III	65 (44.8)	
– IV	70 (48.3)	
– Not stated	1 (0.7)	

NOS: Not other specified; NSCLC: Non-small-cell lung cancer; SCC: Squamous cell carcinoma.

650.0 to 14050.0 pg/ml) and was higher than the median of the control group, 2710.0 pg/ml (values between 1290.0 and 8440.0 pg/ml). Median serum level of VEGF was also higher in patients with lung cancer, 521.0 pg/ml (values ranged from 25.0 to 5171 pg/ml) than in the control group, 209.5 pg/ml (ranging from 4 to 1074 pg/ml). In all patients with lung cancer, serum Ang-2 was significantly correlated with serum VEGF with a Spearman's correlation coefficient of r = 0.325 (p < 0.0001).

• Diagnostic performance of serum Ang-2 & VEGF in NSCLC

The ROC curves were made to evaluate the diagnostic power of the serum Ang-2 and VEGF concentrations isolated and the combination of both markers. (Figure 1A–C). The ROC analysis is performed through the study of the function that links the sensibility with 1 - specificity. The subtended area under the ROC curve (AUC) shows a synthetic index of the overall capacity of the test in differentiating between healthy and ill individuals. The closer the area gets to the unit, the greater its discriminating ability. According to our data, the AUC for Ang-2 is 0.670 (95% CI: 0.57–0.68; p = 0.003) and the AUC for VEGF is 0.693 (95% CI: 0.59–0.79; p = 0.001). We defined a biomarker index ($BI_{Ang\text{-}2*VEGF}$) combining both markers (multiplying each level of Ang-2 for the corresponding level of VEGF in the same patient = Ang-2 × VEGF), and the AUC obtained is 0.739 (95% CI: 0.65–0.83; p < 0.0001). We found that lower cut-off values increased the sensitivity of the assay but at the cost of specificity and vice versa. Using the results of the ROC curves, an analysis was made on the test performance with respect to the different cut-off values (Table 2). The results showed that, for Ang-2, considering the cut-off value of 2710 pg/ml (equal to median value observed in the healthy control group), there is a probability of illness of 87.5%

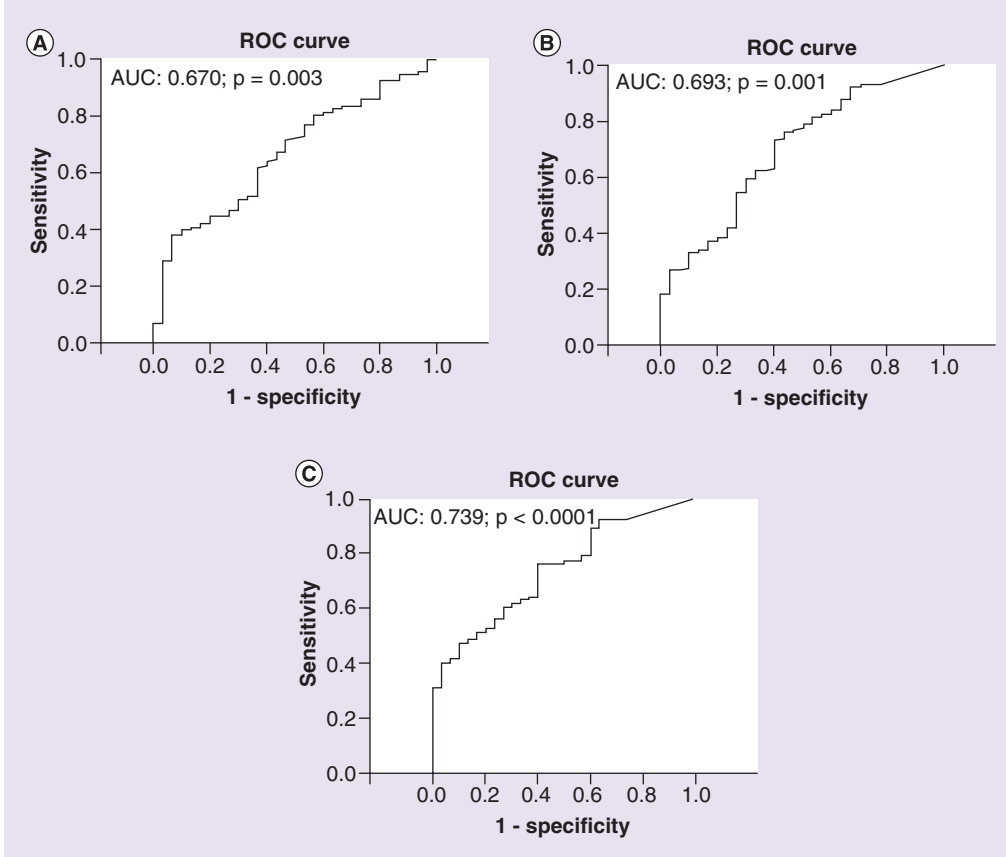

Figure 1. Receiver-operating characteristics curves to calculate sensitivity and specificity of (A) Ang-2, (B) VEGF and (C) combined Ang-2 and VEGF serum levels as tumor markers of non-small-cell lung cancers.

AUC: Area under the curve; ROC: Receiver-operating characteristics.

when the test is positive (PPV). An Ang-2 cut-off level = 2710 pg/ml differentiated between lung cancer patients and controls with a specificity of 50.0% and sensitivity of 72.0%. For VEGF, considering the cut-off value of 209 pg/ml (equal to median value observed in the healthy control group), there is a probability of illness of 88.1%, when the test is positive (PPV). A VEGF cut-off level = 209 pg/ml differentiated between lung cancer patients and controls with a specificity of 50.0% and sensitivity of 77.1%. For the combination of Ang-2 and VEGF levels, with a cut-off level of 566.7×10^3 pg/ml (209×2710 pg/ml), there is a probability of illness of 86.9% when the test is positive (PPV). A $BI_{Ang\text{-}2*VEGF}$ cut-off level = 566.7×10^3 pg/ml differentiated between lung cancer patients and controls with a specificity of 77.8% and sensitivity of 43.3%.

• Serum Ang-2 & VEGF levels & likelihood of presenting NSCLC

To address the question whether or not serum levels of Ang-2 and VEGF are associated with NSCLC, we dichotomized our samples in high and low serum levels subgroups, using the median values of the healthy control group as cut-off points (2710 pg/ml for Ang-2 and 209 pg/ml for VEGF). Therefore, we defined a high Ang-2 group (high Ang-2 >2710 pg/ml) and a high VEGF group (high VEGF >209 pg/ml) and the low serum levels groups (low Ang-2 ≤2710 pg/ml and low VEGF≤209 pg/ml). Moreover, since we observed that serum Ang-2 expression levels were significantly correlated with serum VEGF expression levels ($r = 0.325$; $p < 0.0001$), we grouped all the samples with both high Ang-2 and high VEGF in a different setting (>2710 and >209 pg/ml, respectively), which we termed $high_{Ang\text{-}2/VEGF}$ and compared it with all the other samples regarding the risk of presenting NSCLC.

Our results show that individuals with high Ang-2 or high VEGF serum levels have higher probability of presenting NSCLC than individuals with low serum levels (OR: 2.63, 95% CI: 1.18–5.86, $p = 0.016$; OR: 3.27, 95% CI: 1.45–7.36, $p = 0.003$, respectively). Moreover, we observed that individuals with $high_{Ang\text{-}2/VEGF}$ present an almost fivefold increased likelihood of having NSCLC (OR: 4.66, 95% CI: 1.88–11.5, $p < 0.0001$). Multivariate logistic regression analysis shows that this risk remains statistically significant for the three groups defined, regardless of age, gender and smoking status (Table 3).

Table 2. Test performance for serum Ang-2, VEGF and $BI_{Ang\text{-}2*VEGF}$.

Cut-off value (pg/ml)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Ang-2				
325.0	100	3.3	83.3	100
2430	80.7	43.3	87.3	31.7
2710	72.0	50.0	87.5	27.3
3032.5	64.1	60.0	88.6	25.7
6962.5	13.1	96.7	95.0	18.7
VEGF				
0.5	92.4	23.3	85.3	38.9
150.5	81.9	40.0	86.8	31.6
209.0	77.1	50.0	88.1	31.3
266.5	72.9	60.0	89.7	31.6
858.5	21.7	90.0	92.9	20.5
1069.0	18.1	96.4	96.3	18.6
$BI_{Ang\text{-}2*VEGF}/10^3$				
7.992	92.4	30.0	86.4	45.0
202.3	89.6	36.7	87.2	42.3
566.7	77.8	43.3	86.9	29.6
987.6	62.5	66.7	90.0	27.0
9760	8.33	100	100.0	18.5

NPV: Negative predictive value; PPV: Positive predictive value.

• Serum Ang-2 & VEGF levels & NSCLC clinical outcome

To evaluate the role of serum Ang-2 and VEGF levels in overall survival (OS) of NSCLC patients, we used the same subgroups of the above analysis (high Ang-2 or high VEGF and low Ang-2 or low VEGF serum levels), as well as the $high_{Ang\text{-}2/VEGF}$ setting plotted versus all the other samples (Figure 2A–C).

Our results demonstrate that patients with high Ang-2 serum levels have diminished OS when compared with those with low Ang-2 (21.0 vs 42.6 months, respectively; log rank test, $p = 0.001$) (Figure 2A). There is no statistically significant association between VEGF high and low serum expression levels and OS in our group of NSCLC patients (22.9 vs 30.7 months, respectively; log rank test, $p = 0.619$) (Figure 2B). Interestingly, when comparing the setting of $high_{Ang\text{-}2/VEGF}$ phenotype against all other samples, we observed a decrease in OS in both groups (19.1 vs 35.2 months, respectively; log rank test, $p = 0.005$) (Figure 2C), suggesting that when both Ang-2 and VEGF are elevated in serum, the effect in OS of NSCLC patients is more pronounced than the elevation of each marker by itself.

To determine the independent prognostic value in OS of serum Ang-2 and VEGF expression levels, using the above described approach, a multivariate analysis with Cox proportional hazard

Table 3. Multivariate logistic regression analysis of serum high expression of Ang-2, VEGF and both combined regarding the likelihood of presenting non-small-cell lung cancers.

Serum Ang-2 and VEGF	aOR	95% CI	p-value†
High Ang-2	2.59	1.13–5.93	0.025
High VEGF	3.47	1.49–8.10	0.004
$High_{Ang\text{-}2/VEGF}$	4.56	1.80–11.60	0.001

†p-value, aOR and 95% CI using logistic regression analysis, adjusted by age, smoking status and gender.
aOR: Adjusted odds ratio.

model was performed. In the multivariate analysis that included age, gender, tumor stage, histological type, smoking status and serum Ang-2, VEGF and the combination of both, we identified tumor stage, and high Ang-2 and the $high_{Ang\text{-}2/VEGF}$ as independent prognostic factors for OS in NSCLC patients **(Table 4)**.

Attending to these results, we performed an analysis considering three different prognostic models to ascertain the predictive power of tumor stage, high serum Ang-2 expression levels and the $high_{Ang\text{-}2/VEGF}$ serum levels combined in the clinical outcome of NSCLC patients. In the first model, we considered the predictive ability of tumor stage (c-index 0.657; HR: 2.30; 95% CI: 1.85–2.86; $p < 0.001$; model 1). Model 2 gives the predictive ability of high serum Ang-2 (c-index 0.707; HR: 1.88; 95% CI: 1.23–2.87; $p = 0.003$; model 2). Model 3 addressed the question whether $high_{Ang\text{-}2VEGF}$ serum levels could also be considered a prognostic factor in NSCLC, with a c-index of 0.736 (HR: 1.69; 95% CI: 1.16–2.47; $p = 0.006$; model 3).

Discussion

In recent years, despite major advances in NSCLC therapeutics, its unfavorable prognosis and short median overall survival time have yet to be overcome. The limited tumor response to conventional cytotoxic agents prompted the scientific community to search for alternative therapeutic strategies. The improved understanding of molecular biology of cancer and mechanisms of tumorigenesis have allowed the identification of several potential molecular targets and development of novel targeted therapies [22].

Angiogenesis targeting agents were among the first to be recognized for potential benefit in NSCLC treatment. Several molecules with proved potent antiangiogenic activity in preclinical studies have been developed [16,23], but most of them failed to show efficacy in clinical trials and were abandoned [17]. So far, only Bevacizumab, a humanized anti-VEGF monoclonal antibody, was approved for first-line treatment of advanced NSCLC and has become part of the guidelines of treatment in nonsquamous NSCLC [9]. In spite of what has been learned about its mechanisms of action, suitable biomarkers predicting patients who are likely to benefit from Bevacizumab treatment remain elusive [24,25]. Originally, it was anticipated that traditional markers of tumor angiogenesis would predict outcome to Bevacizumab. However, neither VEGF expression levels nor tumor microvessel density were found to be predictive of treatment response, disease progression or death [17]. Moreover, recent studies suggest that targeting VEGF alone, although effective in eliminating some tumor blood vessels, only temporarily halts tumor growth [26] and that overlapping and compensatory alternative angiogenic pathways provide escape mechanisms that likely limit the full potential of VEGF monotherapies [27,28].

These findings, along with the heterogeneous nature of the NSCLC, its development through a multistep process with potential crosstalk between multiple pathways and the interactions among the tumor and its stroma, leads to the assumption that the best strategy to substantially improve clinical outcome and offer additional clinical benefits seems to be the inhibition of multiple cellular pathways in the tumor and in its milieu [29].

VEGF is the most potent and well-studied proangiogenic signaling factor, primarily produced by tumor cells, and its target structure is the tumor vasculature embedded in the stromal compartment [30]. Ang-2 exhibits a broad expression in the vasculature of human tumors, showing limited postnatal expression in normal tissue (e.g., at sites of vascular remodeling like ovary, placenta, uterus) making it a tumor specific target for antiangiogenic therapies [31]. It has been proposed as a gatekeeper of VEGF, promoting its proangiogenic actions, thereby acting in concert to enhance tumor angiogenesis. While the VEGF and its receptors have been among the most extensively targeted molecules in the angiogenesis field, clinical efforts targeting the more recently discovered angiopoietin-Tie2 pathway are now gaining strength [13,32–34] and interestingly, some preclinical studies suggest that treatment with a combination of Ang-2 and VEGF blockers provides better inhibition of tumor growth than either single agent in a number of tumor models [11,26–27,31,34].

But the main problem subsists, that is, at this time, there are no reliable prognostic or predictive angiogenic markers in the NSCLC population and it is probable that several different biomarkers will

be required to address the unique problems posed by antiangiogenic drugs [35]. Identifying biomarker candidates for prospective evaluation in randomized antiangiogenic trials, for efficacy, safety and cost considerations, remains an outstanding challenge in NSCLC and other cancers [36].

In the present study, we aimed to evaluate the prognostic significance of serum levels of Ang-2 and VEGF in patients with NSCLC prior to treatment. Moreover, due to the mutual influence that both molecules exert in tumor angiogenesis, we wanted to clarify if there is a co-dependent relation in both molecules serum levels and whether the combination of both factors could have a diagnostic and/or prognostic impact in NSCLC patients or in the susceptibility for NSCLC development.

Our results demonstrate that, although isolated serum Ang-2 or VEGF levels are associated with susceptibility to NSCLC cancer, the combination of both yields a more pronounced effect in the risk for NSCLC development. That is, patients with high$_{\text{Ang-2/VEGF}}$ present a higher risk for NCSLC (OR: 4.66; $p < 0.0001$) than when considering Ang-2 or VEGF alone (OR: 2.63; $p = 0.016$; OR: 3.27; $p = 0.003$, respectively). Moreover, we have shown these two markers combined are valuable in discriminating between healthy subjects and NSCLC patients. Our results demonstrate that high$_{\text{Ang-2/VEGF}}$ expression levels are associated with a higher probability of NCSLC presence (AUC for high setting is 0.739; $p < 0.0001$), indicating the ability of these two markers in differentiating healthy individuals from patients with NSCLC.

We also evaluated the role of Ang-2 and VEGF expression levels as prognostic markers in NSCLC and assessed their joint effect in OS of NSCLC patients. Although we studied a Caucasian population, our results are in agreement with a previous study in an Asian population, of Park and co-workers [37], showing that high levels of Ang-2 are associated with a lower OS (21.0 vs 42.6 months, respectively; log rank test, $p = 0.001$) in a univariate analysis. Conversely of Park's results, our study also proved that Ang-2 is a prognostic factor in a Cox multivariate analysis. Moreover, when we consider the setting where both Ang-2 and VEGF expression levels are elevated simultaneously, a significant

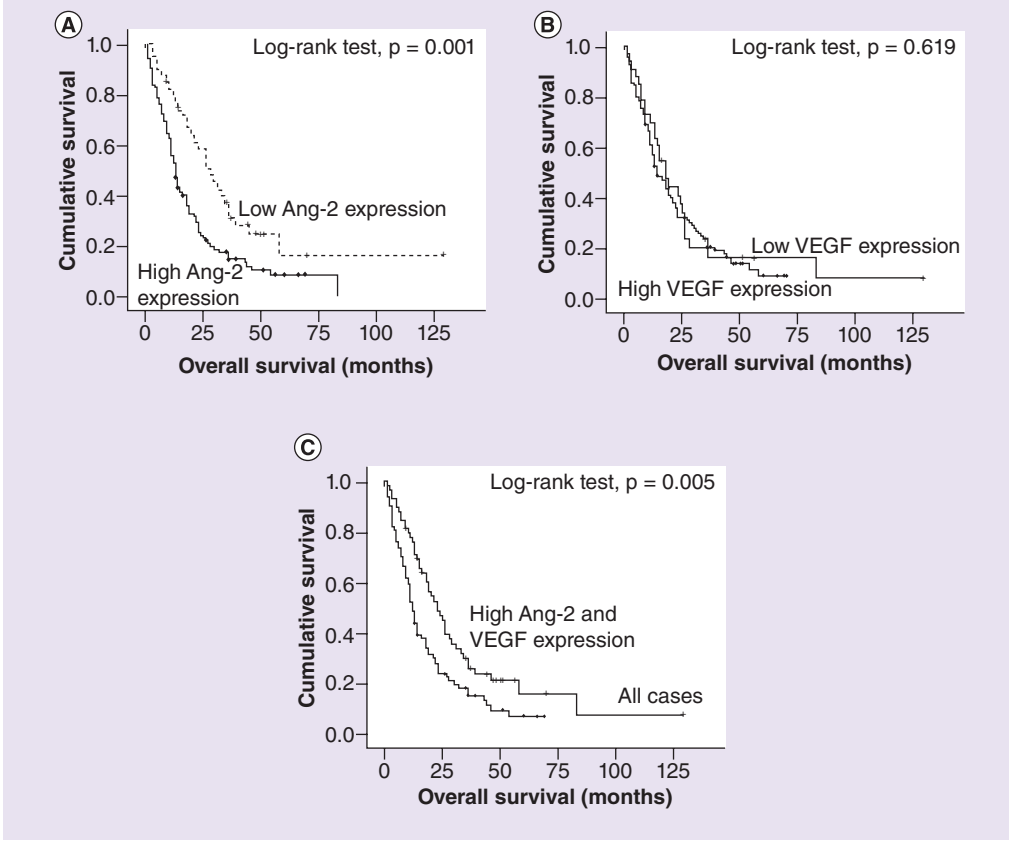

Figure 2. Association of serum levels of Ang-2 (A), VEGF (B) and both combined (C) with overall survival in non-small-cell lung cancers by Kaplan–Meier curves.

Table 4. Multivariate Cox regression model adjusted for predictable factors of overall survival in non-small-cell lung cancers.

Serum Ang-2 and VEGF	aHR	95% CI	p-value†
High Ang-2	1.88	1.23–2.88	0.003
High VEGF	1.16	0.748–1.80	0.509
$High_{Ang\text{-}2/VEGF}$	1.69	1.16–2.47	0.006

†p-value, aHR and 95% CI using Cox regression analysis, adjusted by age, gender, tumor stage, histological type and smoking status.
aHR: Adjusted hazard ratio.

poorer OS was demonstrated when comparing it with all the other patients (19.1 vs 35.2 months, respectively; log rank test, p = 0.005). This result maintains its consistency in a Cox multivariate analysis. $High_{Ang\text{-}2/VEGF}$ independently determines survival, and its prognostic predictive ability (c-index 0.736) is better than for tumor stage or high serum levels of Ang-2 alone (c-index 657 vs c-index 0.707, respectively).

In summary, our results suggest that patients with combined high serum levels of Ang-2 and VEGF can be regarded as a risk group to NSCLC development and bad prognosis.

Conclusion

In an interesting study, Goede *et al.* demonstrated that high serum Ang-2 levels are associated with a poorer response to Bevacizumab, in colorectal cancer patients. Their findings suggest the possibility that patients with high circulating Ang-2 levels may be less responsive to anti-VEGF treatments and might therefore benefit from the combined blockade of Ang-2 and VEGF [24].

In view of our findings and after the proof of concept of Goede's work, it is our belief that the assessment of combined serum levels of Ang-2 and VEGF can be regarded as one step forward in the identification of a new predictive biomarker to be included in future NSCLC preclinical/clinical trials concerning antiangiogenic drugs.

The determination of this noninvasive predictive algorithm (serum levels of Ang-2 plus VEGF) is a very appealing approach, since samples are highly available, techniques are less expensive, readily performed, easily repeated and less biased than the more inconvenient, less practicable assessment in tumor tissue [17,22,38].

Although large-scale prospective studies are needed to confirm this hypothesis, this $high_{Ang\text{-}2/VEGF}$ candidate biomarker should be regarded as a new integral biomarker in NSCLC, and be actively explored in trials of antiangiogenic agents in patients, to get closer to the goal of improving and individualizing cancer therapy.

Future perspective

The advent of targeted agents that inhibit tumor specific biological pathways opened the door for a new era of NSCLC treatment. These include some very promising approaches, directed not only to specific targets in the tumor cells, but also toward the tumor stroma. Among these are the antiangiogenic strategies, where targets have been extensively investigated, with continuous novelties in the field being published in a regular basis. The main focus of antiangiogenic strategies has passed through VEGF and its receptors, but in recent years, Ang-2 has come out to the light. Extensive research presents it as a credible partner/modulator of VEGF in tumor angiogenesis, making it a potential therapeutic target in several clinical trials involving antiangiogenic molecules, and the combination of agents that target simultaneously VEGF and Ang-2 might be more advantageous than targeting either of them alone.

Although antiangiogenic drugs are efficacious in unselected populations, increasing market competition between targeted therapies is likely to drive the growth of individualized treatments, with a central role for biomarkers. Albeit all the progresses made in this area, the quest for a biomarker that can lead the way when choosing the appropriate antiangiogenic treatment continues. We believe that for complex mechanisms such as tumor angiogenesis, there is the need to enlarge the search for biomarkers and that there is not a solo biomarker that can help to predict response, but rather a combination of biomarkers that will increase the chances of successful treatments.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

EXECUTIVE SUMMARY

Background

- The purpose of this study was to evaluate the prognostic relevance of combined serum Ang-2 and VEGF levels in non-small-cell lung cancers (NSCLC) patients and based on the proposed interplay between VEGF and Ang-2, it was also examined if there is a co-dependent relation in serum levels of both molecules in patients with NSCLC and whether the combination of both had a diagnostic and/or prognostic impact in these patients or in the likelihood of developing NSCLC.

Patients & methods

- Study population:
  - The study included 145 newly diagnosed and untreated Caucasian patients with NSCLC, from the North region of Portugal, admitted to the Portuguese Institute of Oncology of Porto.
  - Inclusion criteria were histological or cytological confirmed diagnosis of NSCLC, no previous treatment, no prior oncologic disease, available clinical data and Eastern Cooperative Oncology Group performance status ≤2.
  - The control group consisted of 30 Caucasian healthy individuals from the same geographic area as the NSCLC patients, recruited from the blood donor bank of the Instituto Português de Oncologia–Porto, free of malign, inflammatory and connective tissue diseases.
- Serum Ang-2 and VEGF analysis:
  - Serum levels of VEGF and Ang-2 were measured by means of an ELISA using the Quantikine Human VEGF ELISA kit and the Human Angiopoietin-2 Quantikine ELISA kit, according to manufacturer's instructions;
  - To evaluate its roles as diagnostic and prognostic markers in NSCLC, samples were grouped in high Ang-2 or high VEGF serum levels of expression and low Ang-2 or low VEGF levels. Moreover, patients with high serum levels of both molecules were grouped separately in a high$_{\text{Ang-2/VEGF}}$ subgroup.

Results

- Serum Ang-2 and VEGF in NSCLC patients and healthy control individuals:
  - Serum levels of Ang-2 were significantly correlated with serum levels of VEGF.
- Serum Ang-2 and VEGF levels and NSCLC clinical outcome:
  - High Ang-2, but not high VEGF, is associated with worst disease outcome; when comparing the setting of high$_{\text{Ang-2/VEGF}}$ phenotype against all other samples, there is a marked decrease in OS.
- Serum Ang-2 and VEGF levels and likelihood of presenting NSCLC:
  - Individuals with high Ang-2 or high VEGF serum levels have higher probability of presenting NSCLC than individuals with low serum levels; individuals with high$_{\text{Ang-2/VEGF}}$ present an almost fivefold increased likelihood of having NSCLC, regardless of age, gender and smoking status.

Conclusion

- The assessment of combined serum levels of Ang-2 and VEGF can be regarded as one step forward in the identification of a new predictive biomarker to be included in future NSCLC preclinical/clinical trials concerning antiangiogenic drugs.

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Angiogenesis in NSCLC: is vessel co-option the trunk that sustains the branches?

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ABSTRACT

The critical role of angiogenesis in tumor development makes its inhibition a valuable new approach in therapy, rapidly making anti-angiogenesis a major focus in research. While the VEGF/VEGFR pathway is the main target of the approved anti-angiogenic molecules in NSCLC treatment, the results obtained are still modest, especially due to resistance mechanisms. Accumulating scientific data show that vessel co-option is an alternative mechanism to angiogenesis during tumor development in well-vascularized organs such as the lungs, where tumor cells highjack the existing vasculature to obtain its blood supply in a non-angiogenic fashion. This can explain the low/lack of response to current anti-angiogenic strategies. The same principle applies to lung metastases of other primary tumors. The exact mechanisms of vessel co-option need to be further elucidated, but it is known that the co-opted vessels regress by the action of Angiopoietin-2 (Ang-2), a vessel destabilizing cytokine expressed by the endothelial cells of the pre-existing mature vessels. In the absence of VEGF, vessel regression leads to tumor cell loss and hypoxia, with a subsequent switch to a neoangiogenic phenotype by the remaining tumor cells. Unravelling the vessel co-option mechanisms and involved players may be fruitful for numerous reasons, and the particularities of this form of vascularization should be carefully considered when planning anti-angiogenic interventions or designing clinical trials for this purpose. In view of the current knowledge, rationale for therapeutic approaches of dual inhibition of Ang-2 and VEGF are swiftly gaining strength and may serve as a launchpad to more successful NSCLC anti-vascular treatments.

INTRODUCTION

Cancer is a major health issue, constituting the second leading cause of death worldwide and expected to surpass heart diseases as the leading cause of death in the next few years [1, 2]. In 2013, the incidence of cancer cases worldwide was 14.9 million, with 8.2 million cancer-related deaths. Lung cancer was the most common incident form of cancer, with an estimated 1.8 million new cases having deaths that exceeded those from any other type of malignancy worldwide, accounting for nearly one in five deaths (1.6 msillion deaths in total) [2]. Most lung cancers (~85%) are non-small cell lung cancers (NSCLC) which are divided according to two major histologic subtypes:

the non-squamous carcinomas (mainly adenocarcinomas) and the squamous-cell carcinomas [3]. The parenchyma and the stroma are the two almost-indistinguishable compartments that compose the NSCLC and build up the tumor microenvironment [4]. The stromal cells contribute to the development and expression of certain cancer hallmark capabilities, defined by Hanahan and Weinberg in 2011 [5]. Among these, angiogenesis assumes major importance, since rate-limiting steps in tumor progressions include gaining access to the host vascular system and the generation of a tumor blood supply to obtain oxygen and nutrients, growth factors and hormones [6].

ANGIOGENESIS AND CANCER

While the identification of massive vascularization in tumors dates back to 1863 [7] and the importance of tumor angiogenesis has been recognised since 1908 [8], it was only through the work of Folkman in the early 1970s that the scientific community acknowledge angiogenesis as a potential target to inhibit cancer progression [9-12]. The therapeutic potential of anti-angiogenic strategies boosted this field of research, placing angiogenesis as one of the major hubs of current cancer research.

It is now widely accepted that most tumors and metastases originate as small avascular structures which must induce the development of new blood vessels from pre-existing ones in order to grow beyond a minimum size of 2-3 mm^3 [6, 13]. To achieve this, tumors undergo an angiogenic switch, disrupting the equilibrium between pro and anti-angiogenic regulators and favouring pro-angiogenic mechanisms. Signalling molecules induce quiescent endothelial cells to continuously sprout from existing blood vessels, thereby forming new vessels that help to sustain expanding neoplastic growth [6, 14, 15], according to the conventional model of angiogenesis known as angiogenic sprout [16].

Decades of research investigating the molecular basis of angiogenesis led to the discovery of a number of angiogenic molecules that promote tumor angiogenesis [15]. Of all the identified angiogenic pathways, the most critical appears to be the one involving the VEGF family and their receptors (VEGFR1-2-3) [17-19], although a number of other important molecules and their receptors have also proven to work in combination with VEGF/VEGFR signalling in tumor angiogenesis [19]. These include the fibroblast growth factor receptors (FGFRs) family and their ligands, particularly FGF1 and FGF2, that induce the proliferation and migration of endothelial cells [20]; as well as the platelet-derived growth factor receptors (PDGFRs) and their ligands (PDGFs) that, either alone or in combination with FGF and VEGF, are associated with tumor vascularization in malignant disease, including NSCLC [21, 22] and the Ang-Tie-2 system [19, 22]. Ever since the identification of VEGF as the first endothelium-acting specific cytokine in 1983 [13, 23, 24], its overexpression has been found in several human tumors, including NSCLC [25-29]. More recently, scientists are gaining a better understanding of the many functions of this molecule in the tumor angiogenic process [29]: it triggers multiple signalling networks that enhance endothelial cell proliferation and survival, increases migration and invasion of endothelial cells, increases vascular permeability of existing vessels, and enhances chemotaxis and mobilization of bone marrow derived endothelial progenitor cells (EPCs) into the peripheral circulation [30, 31].

The growing acknowledgment of VEGF's key role in tumor angiogenesis has made it an attractive target for therapeutic intervention in cancer. The VEGF pathway is a promising avenue in research that aims to uncover more effective, targeted anti-angiogenic strategies [23, 32]. The extensive investigation in this field has led to the study of several anti-angiogenic agents, including monoclonal antibodies to block VEGF and its receptor VEGFR2 and VEGFR tyrosine kinase inhibitors (TKIs) [33].

ANTI-ANGIOGENIC THERAPY AND LUNG CANCER

From the multitude of potential therapeutic options that target angiogenesis in NSCLC, [34] (Table 1), there are currently three anti-angiogenic compounds approved by EMA for the treatment of NSCLC. Bevacizumab, an anti-VEGF monoclonal antibody that blocks the binding of VEGF to its high-affinity receptors, was the first angiogenic inhibitor to complete clinical development, showing clinical benefit in patients with metastatic colorectal cancer when combined with chemotherapy [31, 33]. It was approved in 2006 for the treatment of advanced non-squamous NSCLC in the first line setting in combination with chemotherapy [29]. In 2014, ramucirumab, a fully humanized monoclonal antibody that targets angiogenesis by specifically binding to VEGFR-2 with higher affinity than its natural ligand VEGF [35], was approved for the treatment of patients with metastatic NSCLC in second line setting, in combination with docetaxel [36]. In the same year, nintedanib, an oral medication that can simultaneously inhibit triple angiokinase, VEGFR, platelet-derived growth factor receptors (PDGFR), and fibroblast growth factor receptors (FGFR) signalling pathways, was approved to be used in combination with docetaxel in patients with locally advanced, metastatic, or locally recurrent NSCLC adenocarcinoma, after first-line chemotherapy [37]. There are also other potential agents that are under clinical evaluation, whether that be in the clinical trial stage or currently waiting for approval for treatment of metastatic or recurrent NSCLC [38, 39].

In spite of the impressive clinical efficacy of

Table 1: Angiogenesis inhibitors in non-small cell lung cancer (NSCLC)

Approved		
Drug	**Target**	**Indication**
Bevacizumab	VEGF	First-line treatment of nonsquamous NSCLC with CT
Nintedanib	VEGFR 2, FGFR 1-3, PDGFRα and β TKI	Second-line treatment of adenocarcinoma NSCLC with CT
Ramucirumab	VEGFR-2	Second-line treatment of NSCLC with CT
On clinical trials or not approved		
Drug	**Target**	
Vandetanib	VEGFRs, EGFR, and RET	
Sunitinib	VEGFRs, PDGFRs, KIT, FLT3, CSF-1R, and RET	
Aflibercept	VEGF	
Sorafenib	VEGFR, PDGFRs, FGFR, KIT, and RAF	
Motesanib	VEGFRs, PDGFRs, and KIT	
Pazopanib	VEGFRs, PDGFRs, FGFR, and KIT	
Cediranib	VEGFRs	
Cabozantinib	VEGFR, RET, and MET	
Axitinib	VEGFRs, PDGFRs, and KIT	

CT - chemotherapy; VEGF - vascular endothelial growth factor; VEGFR - vascular endothelial growth factor receptor; FGFR - fibroblast growth factor receptor; PDGFR - platelet-derived growth factor receptor; KIT - stem cell factor receptor; FLT3 - Fms-like tyrosine kinase-3; CSF-1R - colony stimulating factor receptor; RET - glial cell-line derived neurotrophic factor receptor; MET - met proto-oncogene;

bevacizumab, ramucirumab, and nintedanib in various cancer treatment settings, the results were relatively modest and limited [40]. In addition, the clinical use of VEGF/VEGFR blockers as anti- angiogenic therapy for patients with advanced NSCLC has been more challenging than anticipated by the preclinical experiments in which long-term benefit of VEGF/VEGFR inhibition was achieved [41]. Anti-angiogenic agents are usually given to all patients for the approved indications; in a high fraction of these patients, however, the tumor is intrinsically refractory to the anti-angiogenic therapy and the disease progresses ceaselessly [42]. Moreover, when there is no intrinsic resistance, acquired resistance to therapy can rapidly occur and limit the efficacy of the anti-angiogenic treatments [41, 43], and the clinical benefit of prolonging cancer patients survival with advanced disease becomes limited, often in the order of weeks or months [16, 44].

Tumor resistance to the anti-angiogenic therapies (whether intrinsic or acquired), represents a significant problem faced in routine clinical practice. The mechanisms underlying the response to these therapies are far from being clearly understood, further fuelling this active field of research [43]. Preclinical investigations have shed some light on the subject, and although different authors propose escape ways from angiogenic inhibitors that are somewhat distinct, some key features appear to be consensual among most of them; these features are likely to be involved in primary and acquired resistance and deserve consideration [16, 18, 41-43, 45]. One of such features is invasive (or metastatic) co-option of normal quiescent vessels without requisite of angiogenesis.

VESSEL CO-OPTION AND LUNG CANCER GROWTH

It is widely accepted that tumor progression is heavily dependent on angiogenesis. Much less understood, is the concept that angiogenesis is necessary for a tumor to become larger than a few millimetres and become clinically detectable, as some research has shown that angiogenesis is not always a pre-requisite for tumor growth [46]. Hence, one possibility for anti-angiogenic therapy resistance is that some primary and metastatic tumors are non-angiogenic, meaning that these tumors do not need angiogenic sprout to obtain an efficient blood supply [47]. Rather, the tumors use alternative vascularization mechanisms. For example, in vessel-dense tissues, the most likely route is hijacking the pre-existing normal blood vessels [42, 44, 48], and more aggressive tumors can undergo vasculogenic mimicry, a process by which tumor cells dedifferentiate to an endothelial phenotype forming structures that provide tumour cells with a secondary circulation system independently of angiogenesis [49].

When tumors arise in well-vascularized organs, their growth will rely on the invasion of host tissue. Enhancement of invasion and metastasis facilitates access to normal tissue vasculature, and cancer cells stay in close contact with the surface of blood vessels [39, 50, 51]. This allows tumor cells to grow and migrate along quiescent normal vessels and take their oxygen and essential nutrients without obligate neovascularization, in

a process known as vessel (or vascular) co-option [42, 43, 49]. These non-angiogenic tumors are a separate group of fast-growing malignancies with little apoptosis and very efficient mitochondrial metabolism [52]. This seems to be the case of tumors arising in the lungs, liver, and brain, areas where this form of vascularization appears to assume a major role [31, 50, 51, 53, 54]. This is also true for tumor metastasis that occurs through lymph and blood vessels and outgrow mostly in these vessel-dense organs [55-58].

In recent years, research related to angiogenesis has been massive; but on the contrary, there is a scarcity of research focusing on tumors that escape pathways of classical angiogenesis and use vessel co-option as an alternative blood supply for tumor growth. This has led to a dearth in information regarding the mechanisms and players involved in that process.

The first insights into the relationship between vessel co-option and lung cancer were made by Pezzella and co-workers, who described NSCLC that grew without morphological evidence of neoangiogenesis but with signs of normal tissue vessel exploitation [59]. They characterized these tumors as having an alveolar pattern, with tumor cell nests filling the alveolar spaces without destruction of the lung parenchyma. The only vessels evident in these tumors appeared to belong to the trapped alveolar septa [59]. Moreover, patients with alveolar pattern tumors presented a worse survival rate than their angiogenic counterparts. Later, when investigating the possible role of microvessel count in NSCLC as a potential marker of disease prognosis, Offersen and colleagues [60] identified the same special vascular pattern in 17 out of 35 NSCLC samples, thus confirming the description of Pezzella's group. Their observations led them to the hypothesis that these alveolar tumors are nonangiogenic and invasive and exploited the pre-existing vascular beds. They also noted that some tumors exhibited only the alveolar pattern while other tumors presented a mixed alveolar pattern consisting of both alveolar and angiogenic features [60]. There was no correlation, however, between angiogenic or vessel co-option status and disease aggressiveness.

Taking into account the NSCLC growth patterns, Nia Sardari *et al.* suggested a modification of Pezzella's classification according to morphological features, based on the biological properties of the tumor-lung interface, which is the region where the tumor expands and the tumor-stroma interactions are more active and homogeneous [61]. According to them, NSCLCs can be classified as having a destructive growth pattern (angiogenic growth pattern), papillary growth pattern (with preservation of the alveolar structure of the lung parenchyma at the interface with co-option of alveolar blood vessels with formation of stromal stalks and subsequent angiogenesis), and alveolar growth pattern (preservation of the alveolar structure of lung parenchyma with co-option of septal blood vessels and without evidence of new stroma formation at the interface). Moreover, they suggested that, in NSCLC, a low degree of ongoing angiogenesis is predictive of poorer prognosis [61, 62].

The hypothesis of co-option by lung metastases, which are often the main cause of death in many solid malignancies, was also proposed by Pezzella's group back in the 1990's. They observed that, regardless of the angiogenic status of the primary breast carcinomas, they could relapse as nonangiogenic tumors in the lungs. This was also true for lung metastases of human renal and colorectal carcinomas [56, 63, 64]. In a very recent study, Szabo and co-workers used cell lines from six different solid tumors, and showed that lung metastases

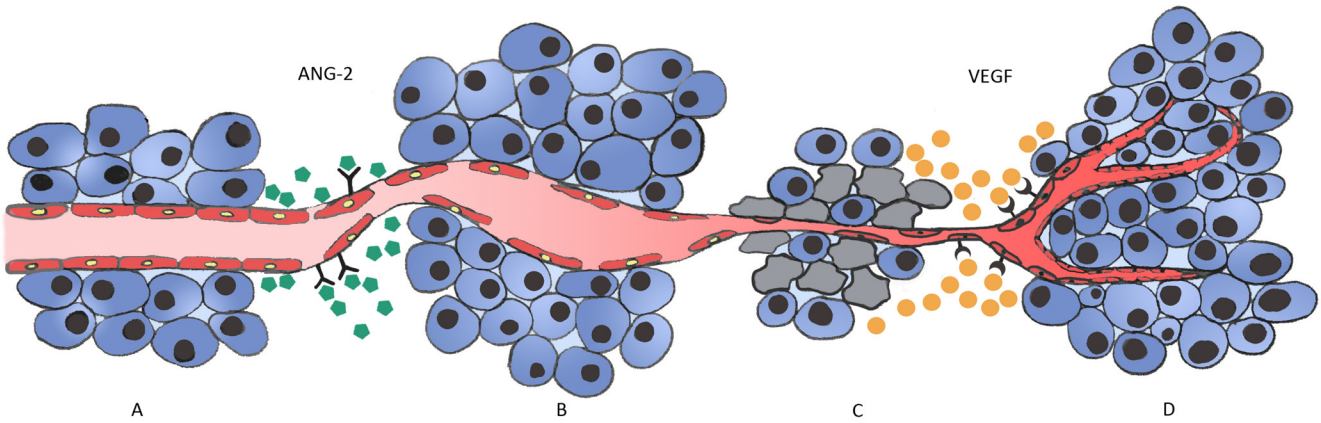

Figure 1: Vessel co-option and Ang-2 regulation in cancer development in vessel dense tissues. A. In well vascularized organs, such as the lung, tumor cells grow and migrate along quiescent normal vessels (vessel co-option). **B.** Over time, tumor cells induce extreme changes in the co-opted vessels and ECs start to express Ang-2, leading to vascular disruption and vessel regression. **C.** Regression of the co-opted vessel associated with regression of the ECs generates a hypoxic core in the tumor centre, with massive tumor cell loss. This triggers the angiogenic switch, with the remaining tumor cells expressing high amounts of VEGF. **D.** VEGF expression induces a robust angiogenic response that ultimately rescues the tumor and allows its growth and progression.

Table 2: Ang-1/Ang-2 and Tie inhibitors in development for NSCLC

Drug	Target	Studies
Regorafenib	VEGFRs, PDGFRs, FGFR, RET, Kit, B-Raf and Tie-2	NCT01187615
Trebananib Fc fusion peptibody	Ang-1 and Ang-2	NCT01666977, EudraCT 2011-001111-31
Foretinib	VEGFRs, PDGFR β, FLT3, MET, and Tie-2	NCT01068587
MGCD265	VEGFRs, MET, and Tie-2	NCT02544633, EudraCT 2015-002070-21
AMG 780 Fully human anti Ang-1/2 mAb	Ang-1 and Ang-2	NCT01137552

VEGFR - vascular endothelial growth factor receptor; PDGFR - platelet-derived growth factor receptor; FGFR - fibroblast growth factor receptor; RET - glial cell-line derived neurotrophic factor receptor; KIT - stem cell factor receptor; Tie - endothelial tyrosine kinase-receptor; FLT3 - Fms-like tyrosine kinase-3; B-Raf - serine/threonine-protein kinase;

vascularize by co-opting the pulmonary microvasculature. The investigated cell lines incorporated the pre-existing host tissue capillaries within the alveolar walls, striping the epithelium from these co-opted alveolar walls [57]. Once there, the metastases expand as the malignant cells spread from one alveolar space to another. Their work not only shed some light on the mechanisms underlying this phenomenon, but it also raised some questions surrounding the biology of the nonangiogenic tumors, further advocating the need for additional exploration in this subject.

VESSEL CO-OPTION AND ANGIOPOIETIN-2

In the lungs, the normal co-opted vessels trapped in the tumor can be very effective because they allow for more efficient tumor growth by exploiting the highly regular vascular network of the lungs and progressively filling the empty alveolar spaces [46]. Regardless of the efficacy of vessel co-option in sustaining tumor growth, the quiescent blood vessels co-opted by tumors suffer extreme changes over time [65]. While there is still debate if this due to a host defence mechanism against tumor development [47] or whether dependence on the survival of endothelial cells (ECs) [50], there is little doubt on the subsequent alterations observed. First, in the centre of the tumor, there is widespread regression of the co-opted vessels associated with the regression of the EC, turning it progressively hypoxic, with subsequent massive tumor cell loss [13, 32], followed by a robust *de novo* angiogenesis at the outer rim of the tumor, that rescues the remaining tumor cells in a later stage [13, 31].

The key regulator in the regression of the initially co-opted blood vessels appears to be Angiopoietin-2 (Ang-2) [49, 53, 66], a cytokine that belongs to the Angiopoietins family, an important class of angiogenic molecules. It is a natural ligand of the endothelial tyrosine kinase-receptor, Tie-2, primarily synthetized and secreted by ECs at sites of vascular remodelling, like tumors, in a tightly regulated fashion [66-68]. Ang-2 is overexpressed in a number of tumors including NSCLC [69, 70], and there is also evidence that it is deeply involved in lung metastases homing and progression [71, 72]. Experimental evidence supports the notion that, soon after vessel co-option, host vessels start to express high levels of Ang-2 that acts through an endogenous autocrine loop mechanism that is context dependent [73, 74]. When it binds to its Tie-2 receptor, it functions as a vessel-destabilizing molecule that converts mature vessels to a tenuous and plastic state by inducing loosening of endothelial cell interactions with pericytes and smooth muscle cells, leading to the loss of vascular integrity and increased vascular permeability. The ECs of such destabilized vessels can be prone to two fates, depending on the local cytokine milieu [74, 75]. In the presence of VEGF, these cells will respond to the proliferating signals induced by the pro-angiogenic molecule and will migrate or proliferate, triggering a sprouting angiogenesis [13, 66, 70, 73, 76, 77]. In the absence of VEGF, however, the expression of Ang-2 causes irreversible loss of vascular structures [76,78] with marked regression of the co-opted vessels, as is the case when tumors co-opt pre-existing vessels [77]. This is due to the fact that, without the pericytes coverage, the ECs of the Ang-2-unstable vessels will die [79] in a very similar fashion to what happens with primitive vessels during development [74]. This generates the hypoxic core and the apoptotic tumor cell loss observed in nonangiogenic tumors [47, 76], that presumably act as the initial stimulus for the molecular changes that culminate in VEGF expression by the remaining tumor cells and in neoangiogenesis [69], mediated both by VEGF and Ang-2 [47] (Figure 1).

Not surprisingly, the discovery of the role of Ang-2 in tumor progression led to the suggestion that its inhibition could translate into clinically meaningful responses, opening the door to multiple approaches that have been used to experimentally inhibit Ang-2 as well as explore its effects on angiogenesis and tumor growth [80-82]. Pre-clinical models revealed that Ang-2 inhibition reduces the growth of a broad range of tumors. Although

some of the results were modest, some revealed to be very promising and there is now a robust pipeline of drugs targeting the Ang/Tie-2 system in different clinical trials phases (Table 2) [67, 78, 83]. Furthermore, with Ang-2 being required to render endothelium responsive to VEGF and with both molecules contributing to tumor angiogenesis and metastases [84, 85], there seems to be a more encouraging response to the straightforward question of whether co-targeting of both ligands in a bispecific manner would improve the outcomes of current anti-angiogenic therapies [80, 83, 86-88].

VESSEL CO-OPTION AND CLINICAL IMPLICATIONS

The ability to identify tumors that make vascular co-option their primary source of blood supply does not envisage an easy task, hence why few strategies have been used to achieve this goal [48]. Research in the field has been scarce, especially when compared to the angiogenic field that has largely overshadowed alternative blood sources for tumor development. Moreover, much of the research has been performed in cell lines or murine models and only a few in human tissues [48]. While the findings are limited so far, what has been discovered highly advocates for unravelling the vessel co-option mechanisms and involved players. The precise identification of tumors that preferentially use this route to support growth and the factors driving them to switch from this to an angiogenic pattern may be crucial to delineate future cancer treatments for two main reasons. The first is that vascular co-option may represent a clever strategy by which tumors partly evade and resist conventional anti-angiogenic treatments [89]. Even if a treatment like bevacizumab is effective against one angiogenic factor such as VEGF, the therapy can still fail if this factor is not important for the endothelium in that given tumor, as appears to be the case in tumors that co-opt pre-existing vessels in NSCLC [45]. In these cases, vessel co-option may serve as a pathologic biomarker for selecting potentially nonresponsive patients [43]. There is also evidence that in some nonangiogenic tumors, cancer cells adapt by migrating more aggressively into normal tissue [42]; and when anti-angiogenic treatments are used indiscriminately, they may contribute to the selection of clones of nonangiogenic cells that will progress with a more aggressive behaviour [89, 90]. These features should be carefully considered when planning anti-angiogenic therapeutic interventions, suggesting the need for tailor-made treatments against such tumors.

Secondly, anti-angiogenic compounds do not affect incorporated pre-existent vasculature or matured tumor vasculature, making targeting existing vessels on which the tumor growth relies, an attractive approach to accomplish tumor regression [91]. This is also of primordial importance in cases of metastases that establish in well-vascularized organs, since vessel co-option may constitute their primary feeding option [57]. Moreover, it can be speculated that in earlier stages of the tumor, the interval that mediates Ang-2 overexpression, co-opted vessels regression, and *de novo* angiogenesis seems to be the perfect therapeutic window for intervention using a dual-pronged approach with Ang-2 and VEGF blockers rather than in more advanced stages of the disease. This issue should be addressed by investigators developing pre-clinical/clinical trials of drugs that target angiogenesis or envisage tumor arrest by anti-angiogenic strategies.

CONCLUSIONS

Anti-angiogenic strategies focusing on VEGF/VEGFR in combination with chemotherapy marked a milestone in the field of cancer treatment, including NSCLC. However, a relevant number of patients are unresponsive or refractory to anti-angiogenic treatments. Some tumors obviate the need to generate angiogenesis by co-opting host mature vessels and growing along them, using them as blood sources. Vessel co-option is a mechanism that may help explain the limited success of anti-angiogenic therapy in these patients in an adjuvant setting.

Thus far, the only growth factors proven to be associated with vessel co-option are VEGF and Ang-2. This lack of information is likely due to the limited number of studies examining this subject. Ang-2 seems to have a particularly critical role in the process, but is also an extremely laborious study topic due to the complexity of its functions and regulation, which are both highly cell context dependent.

Tumors that grow in non-angiogenic fashions through exploitation of pre-existing vessels are non-responsive to anti-angiogenic molecules and raise a number of concerns in terms of treatment. First, little is known about the modifications a neoplastic cell must go through in order to co-opt a blood vessel, which is a huge obstacle for strategies that aim to interfere with this step in tumor progression. Second, once the tumor is committed to vascular co-option pathway, an effective way of blocking tumor progression would be to target existing tumor vasculature; this would require the availability of tumor-vessel specific targeting agents, however, and the few candidates that have been identified so far have failed to prove their clinical efficacy.

All of these concerns reinforce the need for better understanding of the mechanisms and molecular players underlying vessel co-option during tumor development within the proper biologic context. This would not only explore more assertive cancer treatments and help with the identification of tumors where vessel co-option is the growth support (instead of angiogenesis), but could also help identify patients who may be nonresponsive to current anti-angiogenic treatments. Additionally, it could open doors to novel areas of NSCLC research at both the

molecular and microanatomical level.

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CONFLICTS OF INTEREST

The authors declare they have no potential conflict of interests with this research.

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4. GENERAL DISCUSSION AND CONCLUSIONS

Until the late 1990s, treatment of NSCLC followed the simple algorithm of platinum based combination therapy, with or without surgery and/or radiation therapy, depending upon tumor stage, irrespective of histological subtype and without any option for further lines of treatment [115]. The limited tumor response to conventional cytotoxic agents and the short median OS of NSCLC patients impelled the scientific community to search for alternative therapeutic strategies that could have better performances in limiting NSCLC disease progression. This goal was partially achieved when increased knowledge on the molecular biology of cancer and on the complex mechanisms that underlie NSCLC tumorigenesis revealed that lung cancer is not a single entity, but rather a collection of diseases, identifiable by its molecular abnormalities [116]. This constituted the launchpad to the era of personalized medicine and promptly, several potential molecular targets were identified, and novel targeted therapies were developed based on the inhibition of those targets [116].

Angiogenesis is a hallmark of cancer, and agents targeting key molecules in this crucial process for tumor development were among the first to be recognized for potential benefit in NSCLC treatment. The rationale behind the alleged success that angiogenesis hindrance would bring into NSCLC treatment was very straightforward: angiogenesis is stroma related, and therefore, transversal to all types of cancers, and tumors cannot sustain growth without it [10,11,20,117].

The best studied pathway in the generation of a robust tumor vasculature is VEGF and its receptors (VEGFRs) and not surprisingly, the first approved antiangiogenic treatments were bevacizumab and ramucirumab, two compounds that block VEGF and VEGFR2, respectively, administered in combination with cytotoxic chemotherapy [35,39,41,42]. These agents primarily promote stable disease, targeting immature blood vessels, and normalizing tumor vasculature, improving drug delivery to tumor stroma [62,118]. But these treatments, although exhibiting a substantial improvement in NSCLC median OS, felt short on the high expectations generated around them; there is a considerable subset of patients who do not respond or even experience progression under therapy [39].

Insights into the mechanisms that may be responsible for the weak responses to anti-VEGF pathway inhibitors have shown that overlapping and compensatory

alternative angiogenic pathways provide escape routes that probably limit the full potential of VEGF monotherapies [46], and revealed that the proangiogenic cytokine Ang-2 is likely one of the most important regulators of these processes; Ang-2 overexpression is part of an ‘‘angiogenic rescue’’ when VEGF-VEGFR2 signalling is blocked during tumor progression, being responsible for compensatory tumor revascularization and exaggerating malignant tumor progression by increasing local invasion and accelerating metastasis [119-123]. So, it was proposed that Ang-2 inhibitors, both as single agents or in combination with chemo- or anti-VEGF therapy, could efficiently mediate anti-tumor effects, and several experimental models were developed that confirm this theory [101,118].

After extensive bibliographic research, we pictured another important obstacle to the success of anti-angiogenic monotherapies that target VEGF pathways. Several studies suggest that some NSCLC, with more aggressive phenotypes, obviate the need to generate angiogenesis by co-opting host mature vessels and growing along them, in a process known as vessel co-option [51, 134]. Vessel co-option may be a potential explanation as to why approved NSCLC antiangiogenic therapy in many cases does not appear to be as beneficial as initially expected. Mature, non-angiogenic vessels are intrinsically different from the immature vessels that originate from angiogenesis. They are characteristically surrounded by tight pericyte coverage, showing low angiogenic activity, if any. This constitutes a handicap to therapies aiming to halt tumor development through the blockade of VEGF or its receptors, since there is no obvious expression of these molecules in the tumor stroma during the first steps of tumor progression. Intriguingly, it is observed that blocking VEGF signalling increases co-option and growth of satellite tumors in some tumor models [135]. On the contrary, correlative expression of Ang-2 by ECs in the context of tumor vessel co-option supports the concept that this cytokine has a dominant biological role in this form of tumor vascularization [62], and it has been proposed that it is the main responsible for mature vessel destabilizing actions, which, ultimately, generates a hypoxic core in the tumor, that is posteriorly rescued by an increased expression of VEGF, which induces a robust angiogenic response [136].

Assuming the above scenario, it can be speculated that in earlier stages of the tumor, the interval that mediates Ang-2 overexpression, co-opted vessels regression,

and angiogenesis seems to be the perfect therapeutic window for intervention using a dual-pronged approach with Ang-2 and VEGF blockers rather than in more advanced stages of the disease, serving as a launchpad to more successful NSCLC anti-vascular treatments.

The improved efficacy when VEGF and Ang-2-targeting therapies are combined is now solidly established in preclinical models, and several Ang2-targeting drugs are in clinical trials [62], with very encouraging results, as previously exposed [53,64,69,76,87,100-107].

But regardless of the promising success of the new anti-angiogenic approaches, that inhibit both VEGF and Ang-2-associated pathways, an important issue subsists. Considering the high cost of these therapies, specific biomarkers able to discriminate patients for whom therapy with antiangiogenic inhibitors may be most beneficial and the importance of these as prognostic factors in NSCLC are not only a clinical necessity, but are also an economic requirement [124]. Extensive biomarker programs have been built into numerous clinical studies, and originally, it was anticipated that traditional markers of tumor angiogenesis would predict outcome to angiogenic inhibitors. However, neither VEGF expression levels, nor tumour microvessel density (MVD) were found to be predictive of treatment response, disease progression or death [125]. This boosted a forefront of basic and applied vascular biology research, aiming to find alternative biomarkers that may guide future pharmaceutical development to improve antiangiogenic interventions.

The induction of Ang-2 mRNA in tumor endothelium has made Ang-2 a very attractive circulating biomarker of angiogenic activation during tumorigenesis, and some authors addressed the correlation between mRNA and Ang-2 expression in tumor tissue, the protein circulating levels and cancer development and metastasis, although few of them were able to document a correlation between this molecule and disease clinical features or prognosis in lung cancer [126,127,128,129].

Taking into account the well-established correlation between Ang-2 mRNA and Ang-2 expression in the context of tumorigenesis, the present study aimed to evaluate the prognostic significance of Ang-2 mRNA detection in the cell fraction of peripheral blood of patients with NSCLC, prior to treatment, using qRT-PCR. Moreover, it intended to evaluate the possibility of using Ang-2 mRNA levels as an independent

prognostic factor for NSCLC. Our results demonstrate that high circulating Ang-2 mRNA levels are a significantly unfavourable prognostic factor in NSCLC. When considering all NSCLC stages, patients with high circulating Ang-2 mRNA levels present diminished OS when compared to those with low mRNA expression, and this difference is even more notorious when considering only patients with advanced stage disease (with distant metastasis), the most suitable candidates to current antiangiogenic therapies. Moreover, mRNA levels independently determine survival, and its prognostic predictive ability increases when modelled in a simple and easy to apply nomogram with NSCLC staging, patients' smoking status and Ang-2 mRNA levels. Our findings are in agreement with a similar study from Takanami *et al*, which measured Ang-2 mRNA levels in NSCLC tissue, and observed that higher Ang-2 tissue mRNA levels also confer worst disease prognosis [129].

Taken together, our observations prompted us to think that detection and quantification of circulating Ang-2 mRNA in blood samples, along with proper NSCLC staging, could serve as a biomarker of prognosis in NSCLC, as well as a determinant of the angiogenic state of the tumor. This hypothesis is based on the fact that Ang-2 shows limited postnatal expression in normal tissues, but exhibits broad expression and prominent upregulation in the tumor milieu [53]. This high Ang-2 expression is strongly induced by ECs, and regulated at the transcriptional level, through the upregulation of Ang-2 mRNA [62], and hence, circulating Ang-2 mRNA levels theoretically reflect the overall angiogenic activity of the tumor. This biomarker would offer major advantages over tissue based markers, since blood Ang-2 mRNA samples are easily accessible, and allow the ability to carry out continuous, non-invasive assessments over time and most important, do not rely on the availability of adequate surgical or biopsy specimens, so difficult to obtain in NSCLC.

Keeping in mind that Ang-2 and VEGF are inseparable proteins in tumor angiogenic settings, and the assumed relationship between both, the second part of this study aimed to evaluate whether the serum expression levels of these proteins are correlated in NSCLC patients, and if this has an impact in the disease OS. The evaluation of the impact of combined serum Ang-2 and VEGF levels as a diagnostic tool in NSCLC and its relation with the likelihood of an individual to present NSCLC were secondary endpoints of this study.

Similarly to what was previously described by Park and co-workers [128], we found that serum Ang-2 levels inversely correlate with OS, but unlike Park's results, this is also true after a multivariate analysis, adjusted by age, gender, tumor stage, histological type and smoking status. Moreover, serum levels of VEGF alone did not correlate with prognosis in the studied NSCLC population. An interesting finding of our experiments was that grouping the samples with concomitant high levels of Ang-2 and VEGF ($High_{Ang\text{-}2/VEGF}$), seems to confer a cumulative decrease in OS, suggesting that when both Ang-2 and VEGF are elevated in serum, the prognostic effect in the disease is more pronounced than the elevation of each marker by itself. These results are not surprising, given the recognized tight interplay of the VEGF and the Ang-Tie2 pathway in vessel physiology and pathology. So, we propose that both high Ang-2 and $High_{Ang\text{-}2/VEGF}$ serum levels could serve as non-invasive biomarkers of poor prognosis in NSCLC.

In the current era of antiangiogenic targeted therapies, the analysis of circulating factors may not only help to determine prognosis but also direct the use of targeted therapies [130], yielding personalized information on tumor biology and help to predict tumor responses to angiogenic inhibitors. Herein, we propose two different prognostic biomarkers for NSCLC overall survival, high circulating Ang-2 mRNA levels and combined high serum levels of VEGF and Ang-2, which we believe that deserve further exploitation as predictive biomarkers of NSCLC responses to combined antiangiogenic targeted therapies. Circulating predictive biomarker assays would be of great interest in the clinical practice, mainly because of the simplicity of sampling and the future potential of automation of the technical methods for clinical applicability. Moreover, this kind of tests would allow the tracking of tumor state in the course of the disease.

Another interesting finding of our study was that individuals with isolated high Ang-2 or high VEGF serum levels, have higher probability of presenting NSCLC than individuals with low serum levels, and individuals with the $High_{Ang\text{-}2/VEGF}$ phenotype present an almost 5 fold increased likelihood of presenting NSCLC. The conclusions are valid regardless of age, gender and smoking status. In summary, our results suggest that individuals with high serum levels of Ang-2 and VEGF (alone or in combination) can be regarded as risk groups for having NSCLC.

Given the high prevalence of NSCLC and low survival rates, efforts have been focused not only in the development of more efficacious targeted therapies, but also on developing screening protocols for the high risk population likely to develop NSCLC, since early detection of lung cancer is an important opportunity for decreasing lung cancer mortality.

The National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology (NCCN Guidelines) for Lung Cancer Screening provides recommendations for the selection of high risk groups of individuals, candidates for lung cancer screening, performed with low-dose computed tomography (LDCT), based upon the results of multiple randomized trials that assess whether LDCT screening decreases disease-specific mortality [131]. According to these guidelines, LDCT screening should be considered for high-risk individuals who are potential candidates for definitive treatment. Current or past history of tobacco smoking has been firmly established as a risk factor for lung cancer development with a relative risk for lung cancer being approximately 20-fold higher for smokers than for nonsmokers. So, in the NCCN Guidelines, current and former smokers aged 55 to 74 years with a 30 or more pack year history of smoking tobacco are selected as the highest-risk group for lung cancer and are recommended for screening. Former smokers with a 30 pack-year smoking history who quit smoking less than 15 years ago are also included in this highest-risk group [131]. Individuals aged above 50 years and with a history of 20 pack-year smoking and one additional risk factor, such as radon exposure, occupational exposure, cancer history, family history of lung cancer, disease history (COPD or pulmonary fibrosis) with absence of symptoms or signs of lung cancer fall in the same levels recommendation.

However, LDCT scans have low specificity, only 61%, when screening for lung cancer. For approximately every one true positive scan there are 19 false positive scans [132]. This configures a problem in the generalization of lung cancer LDCT screening for psychological and economic reasons. On one hand, a false positive result induces unnecessary anxiety and fear in the screened individual and his family, and on the other hand, it can result in costly unnecessary follow-up interventions, including invasive biopsies or surgical resection [133].

A way to improve the low specificity value that LDCT screening has on its own, would be its association with easy-to-obtain molecular biomarkers of lung cancer risk that could help to define a more refined subset of individuals at higher risk of developing NSCLC. We believe that one of such risk biomarkers could be the presence of the $High_{Ang\text{-}2/VEGF}$ phenotype in high risk individuals. It seems like a suitable candidate because of its association with an almost 5-fold increased likelihood to present NSCLC and considering this is a circulating biomarker, it would be easy to build minimally-invasive, cost-effective tests aiming its detection in peripheral blood samples.

5. FUTURE PERSPECTIVES

Based on the assumption that angiogenesis is a common feature of all tumors and that it is quite a homogeneous process, tumor angiogenic inhibitors have been developed to apply to most solid tumor types, irrespective of the intrinsic biological characteristics of each tumor. As a consequence of this simplified vision of angiogenesis, clinical development of tumor antiangiogenics was not biomarker-based, and the original promise of success of the current antiangiogenic treatments stumbled across a great diversity of mechanisms of resistance in different patient settings.

The most obvious candidate to join VEGF pathway blockade in the quest for improved antiangiogenic strategies is the inhibition Ang-2-Tie2 axis, which is involved in the resistance to Anti-VEGF(R) agents. Based on current data, the combination of Ang-2 and VEGF targeted modalities would yield more effective results than either alone. Furthermore, targeting Ang-2 would add an extra layer of tumor microenvironment effects by inhibiting the recruitment of TEMs that have been shown to aid tumor and vascular growth. Clinical development and validation of these new strategies would receive a great and decisive advantage from the availability of biomarkers to select the patients who, in a given patient population, are more likely to benefit from these new drugs and strategies.

Our results show that high circulating Ang-2 mRNA levels, high serum Ang-2 and the combination of serum high levels of Ang-2 and VEGF, measured before treatment, confer worst OS to NSCLC patients. So, we propose that they can serve as prognostic biomarkers of survival in NSCLC, regardless of treatment. Large-scale prospective studies need to be conducted to confirm our results.

We speculate that these prognostic biomarkers could as well be candidates to predictive biomarkers of response to angiogenic inhibitors. To demonstrate that a biomarker is predictive of treatment benefit, the study requires biomarker status on all patients as well as patients who were treated with the agent of interest and patients not so treated, preferably in the context of a randomized study. Therefore, we propose the inclusion of the measurements of the combined serum levels of Ang-2 and VEGF and of the circulating levels of Ang-2 mRNA in current and future randomized clinical trials of antiangiogenic agents targeting VEGF and Ang-2 pathways, so the predictive role of these biomarkers can be elucidated. To be confirmed, our hypothesis would help to balance efficacy, toxicity and cost of these novel therapies.

The acknowledgement of vessel co-option as an alternative mechanism that solid tumors use to avoid angiogenesis, opened doors to an entirely new and very interesting field of research, that raises many questions and brings few answers. First, little is known about the modifications a neoplastic cell suffers in order to be committed to vessel co-option. Second, if the tumor is committed to the vascular co-option pathway, an effective way of blocking tumor progression would be to target existing tumor vasculature, but currently, there are no mature tumor-vessel specific targeting agents. All of these concerns reinforce the need for a better understanding of the mechanisms and molecular players underlying vessel co-option during tumor development within the proper biologic context. This would not only explore more assertive cancer treatments and help with the identification of tumors where vessel co-option is the growth support (instead of angiogenesis), but could also help identify patients who may be nonresponsive to current antiangiogenic treatments. Additionally, it could unravel novel areas of NSCLC research at both the molecular and microanatomical level.

Finally, we intend to delineate a population based study of lung cancer screening, selecting a sample population with the high risk factors defined in the NCCN screening guidelines and applying the LDCT scans to all of the individuals, and simultaneously determining the combined serum levels of Ang-2 and VEGF, in order to assess the value of $High_{Ang\text{-}2/VEGF}$ as a biomarker of lung cancer presence.

6. REFERENCES

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